2014

Characterizing the Mechanisms by Which Community Associated Methicillin-Resistant Staphylococcus Aureus Influences Keratinocyte Innate Immune Responses During Recurrent Infection

Ashley Lynn Larm
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

CHARACTERIZING THE MECHANISMS BY WHICH COMMUNITY ASSOCIATED METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS INFLUENCES KERATINOCYTE INNATE IMMUNE RESPONSES DURING RECURRENT INFECTION

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

ASHLEY LYNN LARM

CHICAGO, ILLINOIS

AUGUST 2014

ACKNOWLEDGEMENTS

I would like to thank my basic science mentor, Dr. Katherine Radek for taking me into her lab, teaching me valuable lab techniques, and helping me to formulate a project that I was truly interested in. I must also thank my clinical mentor, Dr. Michael Mosier for going the extra mile to allow me to get a better understanding of the clinical side of my project as well as valuable clinical exposure. Additionally, many thanks go out to Dr. John Clancy Jr. and to my committee chair, Dr. Adam Driks, for their continued support and suggestions on my project as part of my thesis committee.

Additionally, I would like to thank The Burn and Shock Trauma Research Institute, particularly my fellow members of the Radek lab: Mari Kishibe, Casey Holmes, Tina Griffin, Sascha Kristian and Louise Lie; who have all been instrumental in my success in the lab by always providing a friendly and cooperative environment as well as valuable suggestions and support even in the worst of times. I would also like to acknowledge the Infectious Disease and Immunology Research Institute, including its co-directors Dr. Katherine Knight and Dr. James Cook, and the graduate program director, Dr. Makio Iwashima, for providing me the opportunity to obtain a unique master's degree that will open up additional professional opportunities for me in the future.

Finally, and most importantly, I thank my family and friends for their continued support and encouragement. Without them, getting this degree would never have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AD	atopic dermatitis
AMP	antimicrobial peptide
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAMP	cathelicidin antimicrobial peptide
CA-MRSA	community-associated methicillin resistant *Staphylococcus aureus*
CD	cluster of differentiation
ChIP	chromatin immunoprecipitation
CFU/mL	colony Forming Units per Milliliter
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
HA-MRSA	hospital-associated methicillin-resistant *Staphylococcus aureus*
HEK	human embryonic kidney
HMGB1	high mobility group box 1
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IL	interleukin
IL17R	IL17 receptor
kD	kilodalton
LDH	lacate dehydrogenase
LL-37	cathelicidin
LPS	lipopolysaccharide
MALP2	macrophage-activating lipopeptide 2
MRSA	methicillin resistant *Staphylococcus aureus*

MSSA	methicillin sensitive *Staphylococcus aureus*
MyD88	myeloid differentiation primary response protein 88
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	normal human epidermal keratinocytes
nm	nanometer
OD	optical density
PBMCs	peripheral blood mononuclear cells
PBP	penicillin binding protein
PBS	phosphate buffered saline
PBST	phosphate buffered saline + 0.05% Tween-20
PVDF	polyvinyl difluoride
RAGE	receptor for advanced glycation end products
RIPA	radioimmunoprecipitation assay buffer
S. aureus	*Staphylococcus aureus*
SCCmec	Staphylococcal cassette chromosome *mec*
SEM	standard error of the mean
TBS	tris buffered saline
TBST	tris buffered saline + 0.05% Tween-20
Th17	T helper 17
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TSA	tryptic soy agar
TSB	tryptic soy broth
TSST-1	Toxic Shock Syndrome Toxin 1
µg	microgram
µl	microliter

ABSTRACT

Introduction

Within the past decade, community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infection has become a major health concern. In human epidermal keratinocytes, *S. aureus* is mainly recognized through the pattern recognition receptor toll-like receptor 2 (TLR2) and its co-receptor, cluster of differentiation 14 (CD14). Cell components or secreted factors from *S. aureus* likely augment the virulence of CA-MRSA by directly suppressing keratinocyte innate immune responses. High mobility group box 1 (HMGB1) is actively released from eukaryotic cells during infection and can then directly bind to TLR2 to induce inflammation. We hypothesize that live CA-MRSA bacterial isolates cause recurrent infections in the skin by interrupting TLR2-mediated inflammation in keratinocytes to a greater extent than non-recurrent community-associated methicillin-sensitive *Staphylococcus aureus* (CA-MSSA) isolates.

Methods

We asked if recurrent CA-MRSA isolates evade human innate immune responses in the skin by suppressing TLR2-mediated pro-inflammatory cytokine secretion by keratinocytes *in vitro*. We compared the effects of secreted factor exposure and live infection with recurrent CA-MRSA isolates to the effects of non-recurrent CA-MSSA on different components of the TLR2 signaling pathway in keratinocytes. A human

immortalized keratinocyte cell line (HaCats) was stimulated with bacterial culture supernatants or live bacterial isolates for 6 hours. Post infection cell culture supernatants were subjected to ELISA to assess the secretion of pro-inflammatory and anti-inflammatory cytokines. Total protein was also extracted from cell lysates post infection and subjected to Western Blot to assess relative protein levels of TLR2 and CD14, as well as for the activation and phosphorylation of NFκB.

Results

Exposure to bacterial culture supernatants from recurrent CA-MRSA isolates resulted in a significant decrease in pro-inflammatory and anti-inflammatory cytokine and HMGB1 secretion from keratinocytes. Interestingly, live infection of keratinocytes with recurrent CA-MRSA isolates only revealed a significant decrease in the secretion of the pro-inflammatory cytokine IL8 by keratinocytes post infection. There were no significant changes in pro-inflammatory TNFα, anti-inflammatory IL10, or HMGB1 secretion from cells post infection. Additionally, no significant changes in overall cytokine secretion were observed from keratinocytes treated with live recurrent CA-MRSA isolates obtained during subsequent infections of the same patient. Recurrent CA-MRSA live infection also did not result in any significant changes in surface receptor (TLR2 or CD14) expression 6 hours post infection or NFκB activation 15 minutes post infection in our system.

Conclusions

We conclude that, under our experimental conditions, recurrent CA-MRSA bacterial isolates do not suppress TLR2-mediated pro-inflammatory cytokine secretion by

keratinocytes by means of cell surface virulence factors. Based on our findings, it is more likely that recurrent CA-MRSA isolates may utilize a secreted virulence factor(s) to cause recurrent infection. This effect could then perhaps cause a decrease in recognition of the pathogen by TLR2 and the host immune system. It is important to further our understanding of the interactions between recurrent bacterial isolates and the human innate immune response in order to develop improved treatment and management for this pathology.

CHAPTER ONE

INTRODUCTION

Within the past decade, the incidence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections in otherwise healthy individuals has increased and has become a major health concern. The increasing frequency of these infections demonstrates the importance of gaining a better understanding of the pathogenesis of CA-MRSA skin infection in order to progress development of novel treatment regimens.

S. aureus is a rapidly adapting organism that utilizes a number of different secreted and cell surface virulence factors to evade host defenses (4, 6, 7, 45). *S. aureus* is recognized by Toll-like receptor 2 (TLR2), a major cell surface receptor present on a variety of human cell types, including keratinocytes. TLR2 requires interaction with one of its co-receptors (TLR1/TLR6/cluster of differentiation 14 (CD14)), ultimately resulting in an increase in the innate inflammatory response to infection via intracellular nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling (6). TLR2 stimulation and production of pro-inflammatory cytokines can additionally lead to the release of high-mobility-group box 1 (HMGB1) (19). Under normal cellular conditions, HMGB1 acts as a DNA-binding protein and transcription factor. Under inflammatory conditions, such as IL6 and TNFα production, this protein can be translocated from the nucleus to the extracellular space, where it can then bind TLR2

(13). This binding interaction initiates further TLR2-mediated inflammatory responses (13) including the secretion of pro-inflammatory (IL6, IL8 and TNFα) and anti-inflammatory (IL10) cytokines by keratinocytes.

We hypothesize that recurrent CA-MRSA isolates evade human innate immune responses in the skin by suppressing TLR2-mediated pro-inflammatory cytokine secretion by keratinocytes. It is possible that patients with recurrent CA-MRSA infections are colonized with bacterial isolates that possess or upregulate specific virulence factors to evade host defenses. This would facilitate the increase in pathogenicity and frequency of recurrence seen with CA-MRSA isolates. We used the secretion of these cytokines post infection as an indication of the TLR2-mediated inflammatory responses during keratinocyte infection *in vitro*. In all experiments, we compared the effects of recurrent CA-MRSA infection of keratinocytes relative to the effects of non-recurrent CA-MSSA and non-recurrent CA-MRSA isolates on downstream TLR2 signaling events in keratinocytes. The goal of this project was to identify possible mechanisms by which recurrent CA-MRSA isolates can evade keratinocyte innate immune responses, leading to their increased pathogenicity, relative to non-recurrent CA-MSSA isolates, and their increased ability to cause recurrent infection.

Overall Hypothesis

Recurrent CA-MRSA isolates evade human innate immune responses in the skin by suppressing TLR2-mediated pro-inflammatory cytokine secretion by keratinocytes post infection.

Specific Aims

Aim 1: Establish whether recurrent CA-MRSA isolates inhibit key interactions between TLR2 and its co-receptors or downstream intracellular NFκB signaling necessary for pro-inflammatory cytokine production to a greater degree than non-recurrent CA-MSSA isolates using an *in vitro* model of *S. aureus* infection in a keratinocyte cell line (HaCats).

Rationale: In preliminary experiments, exposure of normal epidermal keratinocytes (NHEKs) to recurrent CA-MRSA bacterial culture supernatants exhibited less relative pro-inflammatory (IL6, IL8 and TNFα) and anti-inflammatory (IL10) cytokine secretion from keratinocytes than cells that were exposed to MSSA bacterial culture supernatants. Secretion of these cytokines is at least partially mediated by the TLR2 signaling pathway. Since TLR2 is the main recognition receptor of *S. aureus*, perturbations in this pathway could suppress the innate immune response to infection resulting in decreased recognition of the pathogen and therefore, increased pathogenicity of the bacteria.

Hypothesis I: Recurrent CA-MRSA isolates inhibit the binding between TLR2 and its co-receptors, and reduce downstream NFκB activation or translocation as a mechanism to reduce pro-inflammatory cytokine secretion from keratinocytes.

To test this hypothesis, we will:

Aim 1a: Assess the ability of recurrent CA-MRSA clinical isolates to inhibit the protein-protein interactions between TLR2 and its co-receptors (TLR1/TLR6/CD14), a binding interaction that is required for TLR2-mediated pro-inflammatory cytokine secretion post infection.

Rationale: TLR2 requires recruitment and binding of co-receptors for activation. This leads to the translocation of NFκB into the nucleus where it binds DNA and effects gene transcription, including transcription of pro-inflammatory genes.

Aim 1b: Establish whether recurrent CA-MRSA isolates inhibit NFκB translocation from the cytoplasm to the nucleus or activation via phosphorylation, resulting in a decrease in downstream TLR2-mediated pro-inflammatory cytokine release.
Rationale: NFκB translocation from the cytoplasm to the nucleus and nuclear activation via phosphorylation are required for transcription of downstream TLR2-mediated cytokines.

Aim 2: Assess whether recurrent CA-MRSA isolates disrupt HMGB1-DNA binding interactions at pro-inflammatory cytokine gene promoters, inhibit HMGB1 translocation from the nucleus to the extracellular space, or hinder extracellular HMGB1/TLR2 binding to a greater degree than non-recurrent CA-MSSA, as mechanisms to decrease TLR2 mediated pro-inflammatory secretion from keratinocytes.
Rationale: HMGB1 is a multifunctional protein that acts as a DNA binding protein that can be translocated from the nucleus to the extracellular space during infection. In the extracellular space, it can bind and activate TLR2 to further progress the secretion of pro-inflammatory cytokines. Thus, recurrent CA-MRSA isolates may have evolved mechanisms to impair HMGB1 localization or function in keratinocytes as a mechanism to enhance pathogenicity.

Hypothesis II: Recurrent CA-MRSA isolates reduce HMGB1 secretion and HMGB1 binding to TLR2 as a mechanism to reduce pro-inflammatory cytokine secretion by keratinocytes.

To test this hypothesis we will:

Aim 2a: Evaluate the ability of recurrent CA-MRSA isolates to disrupt the binding of HMGB1 to the promoter regions of genes that encode pro-inflammatory cytokines induced by TLR2 activation.

Rationale: HMGB1 is a nuclear DNA binding protein that is able to bind gene segments encoding pro-inflammatory cytokines, resulting in decreased pro-inflammatory cytokine gene transcription.

Aim 2b: Establish whether recurrent CA-MRSA isolates alter the translocation of HMGB1 from the nucleus to the extracellular space during infection.

Rationale: HMGB1 is normally present in the nucleus but can be translocated to the extracellular space in response to infection. In the extracellular space, HMGB1 can bind TLR2 on the cellular surface to further progress the inflammatory response and therefore, increase the production of pro-inflammatory cytokines.

Aim 2c: Assess whether recurrent CA-MRSA isolates inhibit the protein-protein binding between TLR2 and HMGB1, and to determine if the addition of exogenous HMGB1 can reverse the suppression of TLR2-mediated pro-inflammatory cytokine production.

Rationale: HMGB1 is translocated to the extracellular space post infection where it binds TLR2 to further progress the inflammatory response and therefore, pro-inflammatory cytokine production.

Impact: These studies will further our understanding of how CA-MRSA bacteria are able to interact with the human host innate immune system in the skin in order to cause recurrent infection in otherwise healthy individuals. For the first time, we investigated the innate evasion mechanisms not only of recurrent CA-MRSA clinical isolates between patients, but also within the same patient during subsequent recurrences. This study also provides a novel look at the role of HMGB1 during skin infection. It is known that HMGB1 is secreted by activated macrophages and monocytes after injury or infection and that HMGB1 in the skin can facilitate wound healing (81) and cell migration (82). In the context of skin infection specifically, the involvement and mechanism of this protein remains unexplored. Understanding these mechanisms will allow us to create better and more specific targets for drug therapies, and will potentially alter the way that we currently treat recurrent CA-MRSA infections. CA-MRSA is a growing epidemic. Though there have been studies done looking at CA-MRSA, many of them are genomic (3, 68) or epidemiologic (49, 51, 54) in nature. This study provides the first look at possible alterations in the TLR2 signaling pathway in keratinocytes as well as in the localization or function of HMGB1 in the skin to explain the increased virulence of recurrent CA-MRSA bacteria.

CHAPTER TWO

INTRODUCTION – LITERATURE REVIEW

Staphylococcus aureus as a prominent human pathogen

Infections caused by *Staphylococcus aureus* are very important healthcare issues that are increasing in number and severity resulting in increasing morbidity and mortality of patients (70). In the United States in 2003 alone, 11 million doctor's office visits and over 400,000 inpatient admissions were reported to be due to *S. aureus* skin infections (52). These numbers are further increasing due to the emergence of antibiotic resistant strains. Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are estimated to kill about 20,000 hospitalized patients in America each year (53). In 2005 there were an estimated 94,360 invasive diseases and 18,650 deaths due to MRSA in the US alone, which is more than HIV (4). These statistics are rising due to the emergence of more pathogenic community associated MRSA (CA-MRSA) isolates. These facts demonstrate the importance of gaining a better understanding of the pathogenesis of CA-MRSA skin infection in order to take steps toward developing novel treatment regimens.

Approximately one third of the human population is colonized with *S aureus* (15), but in most cases, these pathogens are commensal and do not cause invasive disease. In other individuals, *S. aureus* can be responsible for life threatening infections in several tissues. Host colonization is the first step in the *S. aureus* infection process and usually

occurs through direct skin-to-skin contact with a colonized individual. Though this contact has been identified as a risk factor for colonization or infection, it does not correlate directly with colonization rates (51). The question then of course is, why do so few patients who are colonized with *S. aureus* develop infection? There have been a number of studies done on *S. aureus* virulence factors in attempt to identify a single virulence determinant responsible for this phenomenon (71, 72), but it is more likely that multiple factors are responsible.

S. aureus and Disease

S. aureus isolates are capable of producing a number of secreted and cell surface virulence factors that allow them to avoid host defenses in order to cause disease. Production of these virulence factors is temporally regulated by the bacteria and is dependent on environmental factors such as cell density and energy availability (4). Expression of the virulence genes in *S. aureus* occurs during very particular bacterial growth phases. For example, in early exponential phase, coagulase, protein A, fibronectin, and clumping factors A and B are expressed. Throughout the entire exponential phase, hyaluronidase, δ-hemolysin and entertoxin A are produced. Many of the other key virulence factors of *S. aureus* are expressed during post exponential phase (43). In general, cell surface virulence factors are expressed during exponential growth phase and secreted virulence factors are expressed during stationary phase (79).

Some examples of staphylococcal secreted virulence factors are superantigens, proteases, and staphylococcal complement inhibitor (SCIN) (6). Superantigens can over-

stimulate a T cell response and result in staphylococcal toxic shock syndrome. Bacterial proteases can cleave and inactivate antimicrobial peptides. SCIN binds and stabilizes convertases on the surface of the bacteria in order to inhibit all three complement pathways (6). There are also a number of bacterial cell surface virulence factors that have been identified in *S. aureus.* These include the presence of adherence proteins that bind and inhibit Intracellular Adhesion Molecule-1 (ICAM-1) in order to prevent leukocyte adhesion and diapedesis as part of the inflammatory response (6). There is also a known redundancy in the innate immune evasion factors of *S. aureus* (6, 7). This suggests that a single molecule or structure is not responsible for the increased virulence of CA-MRSA isolates. This increase in pathogenicity is more likely due to a network of factors, potentially involving both soluble bacterial derived proteins and cell surface components.

Strain-dependent effects relating to mastitis outcomes have also been specifically demonstrated, despite the close genetic relatedness between *S. aureus* strains tested (78). For example, there are four restriction modification systems in *S. aureus* that are known to be responsible for the ability of the bacteria to take up foreign DNA and to acquire mobile genetic elements. Particular strains with mutations in these systems are able to more effectively acquire foreign DNA or mobile genetic elements leading to rapid acquisition of virulence genes and therefore, enhanced virulence (78).

The Emergence of CA-MRSA as a Public Health Concern

Methicillin resistant Staphylococcus aureus (MRSA) first emerged as a public health concern in the early 1960s, at which time it was mostly encountered in a healthcare

setting and affected patients with known immune deficiencies (7). MRSA was first reported just two years after the introduction of methicillin as a treatment for *S. aureus* infection (2), indicating a very high rate of antibiotic resistance acquisition of these bacteria (7). Within the past decade, community-associated MRSA (CA-MRSA) infection in patients with no previously identified risk factors has become a major and growing health concern. CA-MRSA first appeared in the early 1990s and has since become endemic in a number of countries, with the highest prevalence in the United States (7). CA-MRSA has most clearly been linked to situations and places where there is a high chance of skin disruption and close physical contact such as sports locker rooms or at daycare centers. The increasing numbers of infections and hospital visits due to CA-MRSA also indicate the use of additional health care resources and additional cost to the patient (54). In one study, an economic simulation model estimated the annual burden of CA-MRSA infections alone to be between 1.4 billion to 13.8 billion dollars per year (55).

CA-MRSA strains have been shown to have enhanced virulence compared to traditional hospital acquired (HA)-MRSA strains which rarely cause disease outside of the hospital setting (7). These strains are known to be genetically different from one another in the sizes of their large chromosome cassettes (SCCmec) (4). HA-MRSA isolates contain larger SCCmec cassettes than CA-MRSA and CA-MSSA isolates because they are resistant to a larger range of antibiotics (26).

Additionally, differences in the production of secreted virulence factors by CA-MRSA and those produced by HA-MRSA and CA-MSSA strains have been demonstrated (26). These investigators hypothesized that these differences were due to

the niche that the bacteria occupied within the host. Since HA-MRSA isolates contain larger SCCmec cassettes, this may require selection by the bacteria to produce only the secreted factors that are necessary for the survival of the organism in that particular environment. An example of this niche dependency is the lower production of toxic shock syndrome toxin 1 (TSST-1) exotoxin by *S. aureus* strains in the menstrual, vaginal mucosa relative to the levels of TSST-1 production by the same *S. aureus* strain isolated from the skin (26). This is due to the fact that this toxin is required for skin survival and causes a great amount of tissue destruction that would be detrimental to the vaginal mucosa, and therefore, bacterial propagation. Differences in the virulence factors secreted by CA-MRSA and CA-MSSA isolates have also been found. These investigators demonstrated that CA-MRSA isolates produce increased amounts of the secreted superantigenic virulence factors TSST-1 and Staphylococcal enterotoxin C (SEC) relative to their CA-MSSA counterparts *in vitro* (26). Though the clinical significance of this finding is currently unclear, it highlights the possibility that expression levels of virulence factors may play a role in the increased virulence of CA-MRSA isolates.

Unfortunately, the clinical diagnosis of CA-MRSA is not well defined. At Loyola University Medical Center, CA-MRSA is classified as such if a MRSA infection is acquired 48 hours or less after hospital admission. If the infection is acquired after this time frame then the infection is classified as HA-MRSA. This diagnosis definition is problematic for a number of reasons. These reasons include the timing of bacterial cultures depending on clinical symptoms or the fact that *S. aureus* can colonize an individual up to years prior to symptomatic infection (29). Additionally, this definition

does not take any patient factors into account, such as chronic illness or recent antibiotic treatment. This means that by default, any other infection is classified as community-associated, even if there are other underlying health complications that could have resulted in the infection. This definition needs to be further defined in order to better address clinical management of *S. aureus* infections.

Innate Immunity and S. aureus

S. aureus has evolved to become very effective at circumventing the host immune system. The interaction of *S. aureus* with the innate immune system is of particular interest because it acts as a first line of defense against pathogens in the body. The first barrier to bacterial infection is the skin. Epidermal keratinocytes make up the majority of the cells in the epidermis and are responsible for maintaining the physical barrier between the internal and external environments. In addition, keratinocytes encode genes for a number of immune modulators including cytokines, chemokines, and antimicrobial peptides that are upregulated during times of infection or cellular stress (83).

There are a number of important microbial defense mechanisms in humans that are known to be essential for clearance of *S. aureus* including antimicrobial peptides (AMPs). AMPs are small cationic peptides that have broad spectrum antimicrobial activity and are secreted by a number of cell types, including keratinocytes. AMP dysregulation has been implicated in a number of inflammatory diseases, such as atopic dermatitis and psoriasis (18). Human skin keratinocytes secrete only low levels of AMPs under healthy conditions, but AMPs are robustly induced in keratinocytes after infection.

AMPs act as both a natural antibiotic and "alarmins" that further amplify the innate immune and wound healing responses (18). MRSA strains have even been found to have increased resistance to the active form of the human cathelicidin antimicrobial peptide (LL-37) relative to the resistance of this AMP by MSSA strains (12). It remains unclear whether patients with CA-MRSA infection are colonized with these AMP resistant strains.

Another important molecule in the study of the interaction between *S. aureus* and the host innate immune system is IL-17 and its receptor, IL-17R. T helper 17 (Th) 17 cells are recruited to the site of an infection after TLR2 activation and release of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and IL-23 (14). Th17 cells also produce IL-17A and IL-17F which then bind the IL-17 receptor on the surface of keratinocytes, leading to the production of AMPs (14). Interestingly, it has been found that human keratinocytes and bronchial epithelial cells produce more AMPs and cytokines in response to IL17 as compared to other cell types, such as fibroblasts (65). This highlights the potential of IL17 to be used in therapies against skin and lung pathogens.

S. aureus interacts with the host immune system through binding of cell wall lipoproteins to host cell surface receptor Toll-like receptor 2 (TLR2). This leads to the activation and translocation of the transcription factor, NFκB to the nucleus to induce an inflammatory response. TLR2, which is expressed by epidermal keratinocytes, has been found to be a very important receptor in *S. aureus* pathogenesis. It has been shown that mice deficient in TLR2 or myeloid differentiation primary response protein 88 (MyD88),

an adaptor protein required for downstream signaling of this receptor, are highly susceptible to infection by *S. aureus* (8). Therefore, CA-MRSA isolates may be able to cause recurrent infection by subverting the TLR2 recognition pathway in keratinocytes, leading to a decrease in downstream inflammation.

The TLR2 Signaling Pathway and Skin Disease

Toll-like receptors are a family of transmembrane proteins that act to recognize pathogenic stimuli in order to elicit appropriate inflammatory responses to infection. In humans, at least 10 different TLRs have been identified and most of them, with the exceptions of TLR3 and TLR4, signal through the MyD88 pathway which eventually triggers NFκB-dependent events (1). In human skin, *S. aureus* is mainly recognized by keratinocytes via TLR2, which also requires the recruitment and heterodimerization with co-receptors TLR1, TLR6 or CD14. Heterodimerization allows these receptors to detect a wider range of pathogenic targets. For example, TLR2 heterodimerizes with TLR6 to recognize diacylated lipopeptides, such as macrophage-activating lipopeptide 2 (MALP2). TLR2 can also heterodimerize with TLR1 to recognize triacylated lipopeptides (9). This receptor then signals through the MyD88 signaling pathway, ultimately resulting in an increase in translocation of NFκB to the nucleus. NFκB activation results in production of pro-inflammatory cytokines, chemokines, antimicrobial peptides (AMPs), and adhesion molecules (6) (Figure 1).

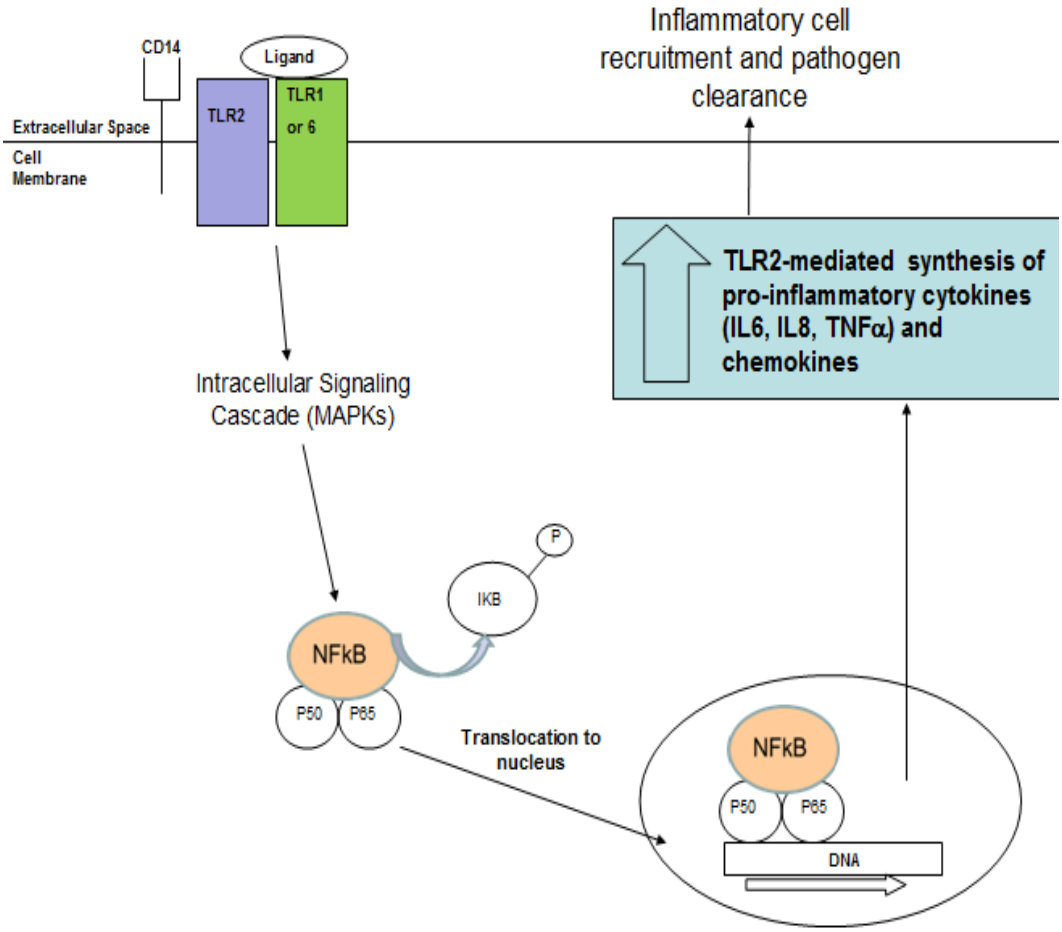


Figure 1: Summary of TLR2-mediated innate inflammatory responses in keratinocytes. In human skin, *S. aureus* is mainly recognized through Toll-like receptor 2 (TLR2) which becomes activated and signals by binding to its co-receptor, CD14 and acting through the MyD88 signaling pathway, ultimately resulting in an increase in translocation of NFκB to the nucleus. NFκB signaling results in production pro-inflammatory cytokines, AMPs, chemokines and adhesion molecules (6).

Alterations or deficiencies in different aspects of innate immunity, including TLR2-mediated mechanisms, are known to be associated with different inflammatory or infectious skin disease states. For example, patients with atopic dermatitis (AD) have been shown to exhibit significantly decreased numbers of TLR2+ peripheral blood mononuclear cells (PBMCs). This suggests that the deficiency of TLR2 in AD patients leads to disease pathogenesis (35). Additionally, the balance between pro-inflammatory

and anti-inflammatory cytokines is crucial to appropriate immune responses. If this balance is disrupted, it can lead to variable levels of inflammation that could be detrimental to the host during infection or disease and therefore, lead to increased symptoms. For example, significantly decreased levels of cutaneous IL10 mRNA expression have been identified in psoriasis patients (73). Administration of subcutaneous IL10 was effective in reducing symptoms of psoriasis in a small number of patients (73), suggesting that at least a subset of psoriasis symptoms are due to the absence of anti-inflammatory cytokines. It is possible that recurrent CA-MRSA isolates are able to suppress the TLR2 signaling pathway, and therefore downstream pro-inflammatory and anti-inflammatory cytokine production, leading to an increase in inflammation, disease symptoms, and/or recurrence.

HMGB1, Inflammation, and Disease

TLR2 stimulation and production of pro-inflammatory cytokines can additionally lead to release of another nuclear protein, high-mobility-group box 1 (HMGB1). HMGB1 is a ubiquitously expressed and highly conserved chromatin binding factor that acts in the nucleus to bend DNA and promote protein assembly during normal cellular conditions. The chromatin is altered by different acetylation patterns in order to allow for the binding of HMGB1; HMGB1 itself remains unaltered (13). Additionally, this protein is secreted by activated macrophages and monocytes under inflammatory conditions and is also known to be passively released from necrotic cells (13). Necrotic cells that are deficient in HMGB1 have a decreased ability to promote inflammation and mice with HMGB1

deficiency die within a few hours after birth (13). Apoptotic cells retain their HMGB1, while necrotic cells release it, which may be a safeguard for the body to prevent unnecessary inflammation (13).

HMGB1 is able to bind either the receptor for advanced glycation endproducts (RAGE), TLR2 or TLR4 differentially between cell types in order to initiate further inflammatory pathways in a systemic manner (Figure 2). HMGB1 binding to RAGE is known to be involved in chemotaxis and cell migration, while interactions with TLR2 or TLR4 are known to be associated with increased cytokine production in response to cellular stimuli (41). HMGB1 activity is associated with the severity of inflammatory disease (1) and has been implicated in a number of severe inflammatory diseases including systemic lupus erythematosus (34), rheumatoid arthritis (31), and ankylosing spondylitis (33). Additionally, high levels of HMGB1 are detected in the circulation of patients with severe sepsis (42). These facts emphasize the importance of understanding the role of HMGB1 in the scope of the immune response to injury and infection.

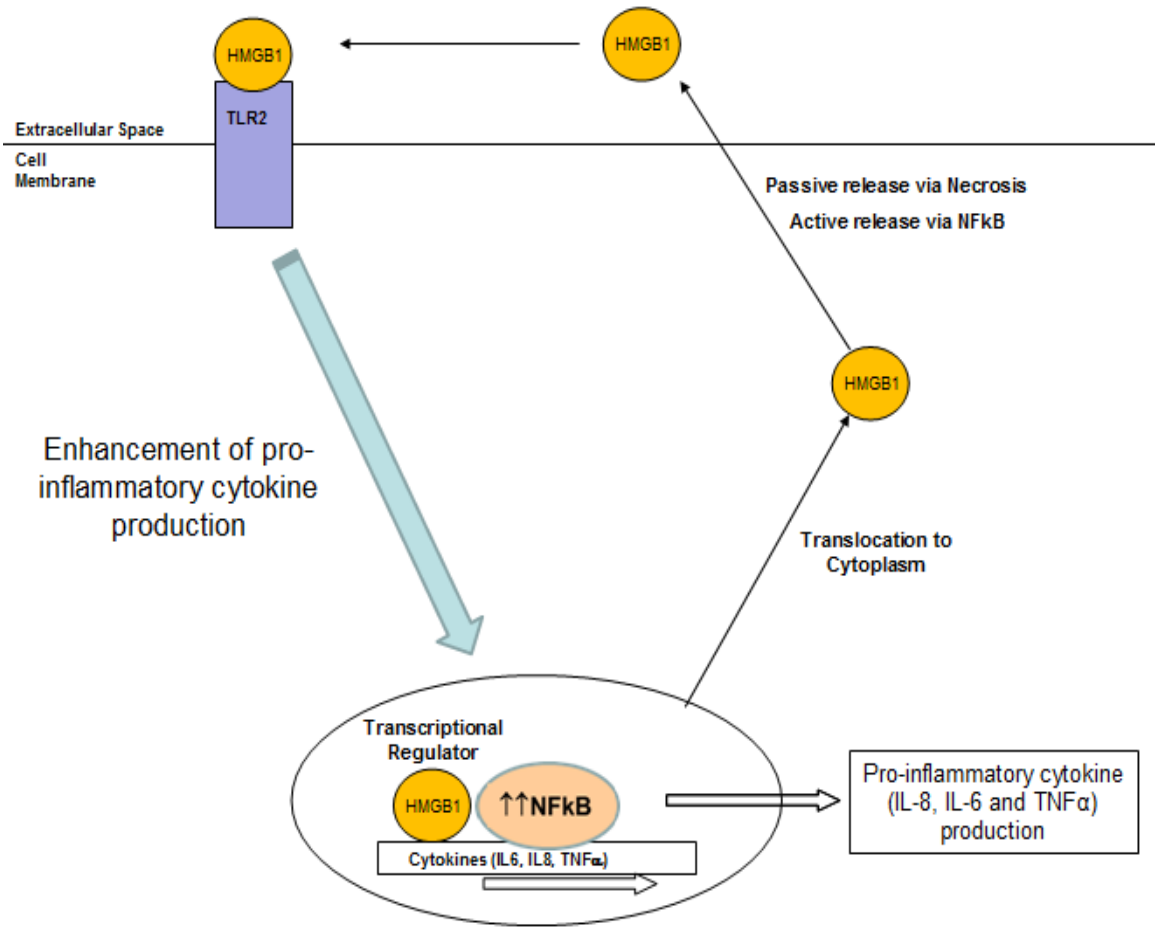


Figure 2: Summary of HMGB1-mediated innate inflammatory responses in keratinocytes. TLR2 stimulation and production of pro-inflammatory cytokines can additionally lead to release of the nuclear protein, HMGB1, from late phase necrotic cells upon infection. HMGB1 is known to be a transcriptional regulator in the nucleus that affects the transcription of pro-inflammatory genes in part by NFκB signaling. HMGB1 can be translocated to the cytoplasm and then to the extracellular space where it is then able to bind to TLR2 in order to initiate further inflammatory pathways in a systemic manner (1).

MRSA Diagnosis and Treatment

S. aureus infections were initially treated with penicillin and then by methicillin after penicillin resistance of these bacteria was identified. Just two years after methicillin began being used, MRSA was identified. MRSA is resistant to all β-lactam antibiotics. In

MSSA bacterial isolates, there are four penicillin-binding proteins (PBPs) that are inactivated in the presence of β-lactams. This causes cell death and inhibition of cell wall biosynthesis (3). MRSA strains have an extra PBP, with a low affinity for β-lactams, which retains its activity even in the presence of β-lactam drugs. This allows for cell wall biosynthesis to continue (3).

Currently, MRSA (including CA-MRSA) infections are treated with a variety of other classes of antibiotics but are continuing to acquire resistance to these antibiotics as well. For example, heteroresistant vancomycin-intermediate *S. aureus* (hVISA) has become an increasing complication in the treatment of MRSA. This resistance pattern is mediated by the expression of a particular gene in some *S. aureus* strains that modifies the structure of the peptidoglycan. This modification causes a loss of affinity of vancomycin for the peptidoglycan precursor (1). Sub-curative doses of antibiotics are indicative of the antibiotic levels in a patient who does not follow a consistent treatment regimen. These antibiotic doses are known to cause clonal expansion of resistant bacteria *in vivo*. This allows them to out-compete antibiotic sensitive bacteria (50).

Due to the fast acquisition of antibiotic resistance of *S. aureus*, a number of different approaches can be studied in order to find new possible treatments for the disease including immunomodulatory and vaccination strategies. One of the possible methods that have been suggested is the activation of TLR2 with an agonist to allow for increased inflammasome activation post infection (14). Another method could be to create an intranasal vaccine against Th17 cell-inducing *S. aureus* antigens to promote Th17 cell activity and Th17 mediated-cytokine production (14). Due to its rising

prevalence, it has become increasingly important to understand how CA-MRSA interacts with the host immune system. This understanding is crucial in being able to efficiently move toward administration of immune based therapies to treat *S. aureus*, including recurrent CA-MRSA infections.

CHAPTER THREE

MATERIALS AND METHODS

Bacterial preparation

For all live infections, we used a protocol adapted from Kisich, et al (21). Bacterial isolates were grown overnight in 3 mL of tryptic soy broth (TSB) at 37ºC with shaking. The next day, 150 µl the bacterial overnight cultures were reinoculated into 3 mL of new TSB media and grown up to a concentration of 1.0 x 10^8 (colony forming units per milliliter) CFU/mL as determined by optical density (O.D) readings measured on a Spectromax spectrophotometer. Using a plate blank of TSB media and the path check function on, OD reads were taken until they were in the appropriate range of 0.75-1.0. The target OD read was 0.75 which corresponds to a bacterial concentration of slightly over 1.0 x 10^8 CFU/mL to allow for some loss of bacteria in subsequent wash steps. If the OD read was greater than 1.0, the bacteria were diluted appropriately with TSB until the read was in range. These bacteria were then diluted appropriately to an OD read of 0.75 and the final volume was brought up to 1 mL with additional TSB. These samples were then spun down at 4000 RPM for 10 minutes, media was decanted, and 1 mL of sterile 1 X phosphate buffered saline (PBS) was added to each sample prior to a quick vortex. This cycle was repeated once more before finally resuspending the bacterial pellet is 1 mL of sterile high glucose, serum free, and antibiotic free Dulbecco's Modified

Eagle Medium (DMEM) containing sodium pyruvate to give a final working stock with a concentration of approximately 1.0 x 10^8 CFU/mL. These bacterial stocks were also serially diluted and plated onto tryptic soy agar (TSA) plates in order to back calculate the concentration of each isolate used in the experiments.

HaCat cell preparation

Human HaCat keratinocyte cells were grown up in DMEM with 10% fetal bovine serum (FBS) and 1x penicillin/streptomycin. The media was changed the day after splitting of the cells as well as every other day until they reached the appropriate level of confluency depending on the experiment. The night before treatment, the media was removed and all cells were washed twice with sterile 1X PBS and serum free and antibiotic free DMEM were then added in the appropriate volumes to the cells for use in experiments.

HaCat cell live infection

Appropriate volumes of 1.0 x 10^8 CFU/mL stocks of each bacterial isolate were be added to the appropriate size cell culture dishes containing HaCat cells and serum free and antibiotic free DMEM for a final infecting concentration of 1.0 x 10^6 CFU/ml and allowed to incubate for an assay dependent period of time at 37ºC and 5% CO_2.

Total protein isolation

After the appropriate incubation time, all cell culture supernatants were removed and the cells were washed twice with sterile 1X PBS. Then an appropriate amount of radioimmunoprecipitation assay (RIPA) buffer + Triton X-100 + Pierce HALT protease/phosphotase inhibitor (Ca. No. 78443) were added to each cell culture dish to

lyse all cells. These plates were then kept at 20ºC prior to cell scraping to remove all cells from the cell culture plate. Sonication of all cellular contents was carried out for 10 seconds to break up the cell membranes prior to collection of the cellular supernatant.

BCA Protein Assay

Total protein levels were determined in cell lysates by utilizing Pierce's Bicinchoninic acid (BCA) protein assay kit (Ca. No. 23227) per the microplate procedure provided by the manufacturer. 25 µl of each unknown sample and bovine serum albumin (BSA) standard (working range 20-2000 ug/ml) were added to a clear 96-well microplate. Then 200 µl of alkaline working reagent containing BCA was added to each well to chelate the Cu+2 ions produced from the reduction of Cu+1 by protein in the alkaline media resulting in a purple color that can be read on a plate reader at 562 nm after 30 minute incubation at 37C.

LDH Cytotoxicity Assay

Post infection cell culture supernatants were used to carry out a lactate dehydrogenase (LDH) cytotoxicity assay per manufacturer's instructions (Abcam, Ca. No. ab65393) to assess the relative levels of cell death between treatment groups and a vehicle control at various time points between 2 and 24 hours post infection.

Cytokine ELISAs

Cell culture supernatants were collected after 6 hours, spun down at 16000g for 10 minutes to remove any residual bacteria, and used to run ELISAs for the pro-inflammatory cytokines IL6, IL8 and TNFα as well as for the anti-inflammatory cytokine IL10 using Peprotech ELISA kits per the manufacturer's instructions.

HMGB1 ELISA

Cell cultures supernatants were collected from cell culture dishes 6 hours post infection with recurrent CA-MRSA, CA-MSSA or control groups. These supernatants were then spun down at 16000g for 10 minutes to remove any residual bacteria and total protein was isolated and quantified via BCA protein assay (outlined above). Supernatants were then used to carry out Biotang's HMGB1 ELISA (Ca. No. HU8317) to look at secreted HMGB1 from keratinocytes in culture between groups per the manufacturer's instructions.

Western Blots

25-40 μg of protein was loaded to a 12% Tris-HCl gel and ran at 90 volts for about 1.5 hours. The gel was then transferred to a polyvinyl difluoride (PVDF) membrane via wet transfer using Biorad tris/glycine buffer + methanol for either 1 hour at room temperature at 100 mV or overnight at 4ºC at 90 mAMPs. The membranes were then blocked with either PBS + 0.05% Tween-20 (PBST) or TBS + 0.05% Tween-20 (TSBT). Next, the membranes were probed with one of the following primary antibodies overnight in block buffer at 4ºC: Rabbit anti-human TLR2 (Abcam, ab108998) at 1:1000 overnight in 5% milk in PBST, Mouse anti-human CD14 (Santa Cruz, UCH-M1) at 1:1000 in 5% milk in PBST, Rabbit anti-HMGB1 (Abcam, ab18256) at 1:1000 in 5% milk in PBST, Rabbit anti-NFκB (Cell Signaling, D14E12) at 1:000 in 5% BSA in TBST, Rabbit anti-phospho-NFκB (Cell Signaling, 93H1) at 1:1000 in 5% BSA in TBST, Mouse anti-β-actin (Santa Cruz, C4) at 1:1000 in 5% milk in PBST, Mouse anti-TATA binding protein (Millipore, 05-1531) at 1:1000 in 5% milk in PBST, Rabbit anti-TLR1

(Abcam, ab68153) at 1:1000 in 5% milk in PBST, or Mouse anti-phospho-IκB (Cell Signaling) at 1:1000 in 5% milk in TBST.

Membranes were then washed 4 times with PBST and probed with an anti-rabbit or anti-mouse secondary antibody (Vector) as appropriate at a 1:5000 dilution in block buffer. The blot was then stripped and reprobed as necessary up to 3 times for each blot. Densitometry was done and all bands were normalized to a loading control in order to evaluate relative levels of target proteins between recurrent CA-MRSA, non-recurrent CA-MSSA or control treatment groups.

TLR2 immunoprecipitation (IP)

Total protein concentration in each sample was determined by a using a colormetric Pierce BCA assay as described above. For the IP procedure, we begin with ~500 μg total protein. Pre-clearance of the samples with 1.5 μl of Rabbit IgG and 20 μl of protein A/G beads was utilized to remove any non-specific binding that may have occurred due to the use of the protein A/G beads. After 30 minutes on ice, these samples were be spun down at 12,000g for 5 mins at 4º C and the supernatants were transferred to a new tube for use in the experiments. The total protein was then assessed again in the same way and the maximal amount of protein from each sample was incubated with rabbit anti-human TLR2 antibody (EPNCIR133) at a concentration of 1:100 overnight at 4ºC with rocking. The next day, 20 μl of protein A/G beads was added to each tube and incubated at 4ºC with rocking again for 1 hour. The samples were then spun down again at 17,000g for 5 mins at 4ºC prior to supernatant collection. The pellet containing the bead/antibody complex was washed twice with 1X PBS, resuspended in 3x protein

sample buffer, boiled for 5-10 minutes, and put on ice shortly. Appropriate samples were quick spun to pellet the agarose beads and the supernatant was loaded to a 12% Tric-HCl gel. The gel was transferred to a PVDF membrane via wet transfer at for either 1 hour at room temperature at 100 mV or overnight at 4ºC at 90 mAMPs. The membranes were then blocked with Easy Blot blocking buffer (GenTex) for 30 minutes at room temperature and then probed with Rabbit anti-human TLR2 at 1:1000 overnight in block buffer at 4ºC and probed with an anti-rabbit secondary antibody (Vector) at a 1:5000 dilution in block buffer. The blot was then be stripped and reprobed for TLR2 co-receptors (TLR1, TLR6 and CD14) subsequently to assess relative binding of TLR2 to its co-receptors between groups. Densitometry was done normalized to total TLR2 and a loading control in order to evaluate relative levels of TLR2 interaction with each of its co-receptors between recurrent CA-MRSA, non recurrent CA-MSSA or control treatment groups.

Immunocytochemistry (ICC)

HaCat cells were grown up to ~60% confluency in 8 well chamber slides and infected with different bacterial isolates in the same fashion as mentioned previously at a concentration of 10^6 CFU/mL and incubated at 37ºC for 6 hours. Supernatants were then removed and cells were washed twice with 1x PBS and then washed six times with 500 µl of 1x Dako wash buffer. The wash buffer was then removed and the cells were fixed to the slide in 500 µl of acetone for 15 minutes at RT with shaking. After removal of acetone, the cells were washed three more times with PBS for 5 minutes each and then incubated in 200 µl of 10% FBS for 30 minutes at RT with shaking. The FBS was then

removed and the primary antibodies were applied to the cells and kept at 4ºC overnight with shaking. The next day, the slides were washed six times with Dako wash buffer prior to incubation with a 1:500 dilution of the fluorescent secondary antibody (FITC conjugated secondary antibody for CD14 or TLR1 and a Cy3 conjugated secondary antibody for TLR2) for 1 hour at RT. The slides were then washed 3 more times with Dako wash buffer and then cover slips were mounted with ~40 µl of Prolong Gold (DAPI nuclear stain) and dried overnight. A species matched normal IgG was used as a negative control to make sure that there is no non-specific binding of the primary antibody resulting in background florescence. The slides were then imaged the next day on the Evos microscope.

Nuclear/cytosolic protein isolation

HaCat cells were incubated for an assay dependent time point with 1.0 x 10^6 CFU/mL of bacteria diluted in serum free and antibiotic free DMEM. After incubation, the supernatant was removed and the cells were washed twice with 1x PBS prior to the addition of 500 mL of 1x PBS + 5 µl of HALT protease/phosphatase inhibitor to each cell culture dish. Then isolation of nuclear and cytosolic protein extracts was carried out using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific Cat No 78835) per the manufacturer's instructions.

Addition of exogenous HMGB1

HaCat cells were infected *in vitro* and 10 ug/mL of exogenous HMGB1 was added at 0 hours post infection with recurrent CA-MRSA or CA-MSSA strains and incubated for 6 hours at 37ºC and 5% CO_2.

Chromatin-immunoprecipitation assay (ChIP)

HaCat cells were grown up to 90% confluence in a 100 mm cell culture dish in 8 mL of DMEM media. Cells were trypsinized with 1 ml of trypsin and 9 ml of DMEM media added for a total volume of 10 mL. 275 µl of a 37% Formaldehyde solution was added and the samples were incubated for 15 minutes with rocking. 1 ml of 1.25 M glycine solution was added to stop the reaction prior to centrifugation at 1500 RPM for 5 minutes at 4ºC. The pellet was then washed with 1 ml of ice-cold PBS and transferred to a small eppendorf tube and spun again at 1500 RPM for 5 minutes at 4ºC. The next day, 500 µl of a cell lysis buffer with protease inhibitor was added to the pelleted cells and incubated on ice for 15 minutes with frequent vortexing. Samples were then centrifuged at 800 g at 4ºC for 5 minutes. The supernatant was removed and discarded and the pellet was resuspended in 500 µl of nuclear lysis buffer plus protease inhibitor. Samples were then sonicated five times at power output 4 for 10 seconds each pulse to produce DNA fragments between 200 bp and 1 kb in size with the majority of them around 500 bp. For each IP sample, 100 µl of chromatin were diluted in 900 µl of dilution buffer and protease inhibitor and pre-cleared with 60 µl of protein G agarose for 4ºC for 5-6 hours. The supernatant was collected via centrifugation and 10 µl saved as input for each sample. Either an IgG control or antibodies specific for pro-inflammatory genes along with 20 µl of magnetic beads was added to the remainder of the supernatant and incubated overnight at 4ºC with rotation. Samples were then spun down and put into a magnet that beads will attach to, the liquid was then removed and the complex was pelleted by brief centrifugation and washed sequentially with low salt buffer, high salt buffer, LiCl buffer

and TE buffer. Chromatin was then eluted from the beads with 100 µl of elution buffer (SDS, water and $NaHCO_3$) and DNA-protein cross-links were reversed with 8 µl of 5M NaCl followed by 65ºC incubation for 2 hours followed by a 95ºC incubation for 10 minutes prior to brining the samples to RT. The samples were placed on the magnet again, the supernatant was harvested and the beads were discarded. DNA purification was then carried out using an Ultraclean kit for DNA purification (Bioexpress #G-3148-250). DNA isolated was then analyzed by qPCR and all samples were normalized to the amount of input DNA.

Statistical Analysis

Cytokine Secretion from NHEK cells during our preliminary experiments was analyzed via one way ANOVA with a Bonferroni multiple comparison post test where a P value of .0001 or less was considered significant. All recurrent CA-MRSA isolates were compared to the MSSA group to determine significance in these experiments. Since the cytokine secretion from HaCat cells exhibited a large degree of variability, we compared the medians of the CA-MSSA and recurrent CA-MRSA groups and analyzed these differences using a Mann Whitney U test for significance in order to account for the non-normal distribution of data produced by the use of clinical isolates. A p value of 0.05 was considered to be statistically significant. When comparing cytokine secretion between different bacterial isolates from the same patient, we utilized the means of these values from 2 independent experiments and represented these in bar graph form indicating the standard error of the mean (SEM). Finally, in analyzing Western Blot data,

densitometry values between two independent experiments were averaged and presented in bar graph form indicating the SEM.

CHAPTER FOUR

EXPERIMENTAL RESULTS

Cytokine Secretion from NHEK cells post exposure to bacterial culture supernatants

It is known that *S. aureus* is most commonly recognized by host TLR2 surface receptors that signal through the MyD88 signaling pathway, ultimately leading to progression of an inflammatory response. We asked if recurrent CA-MRSA isolates secrete a bacterial factor(s) that is able to suppress TLR2-dependent pro-inflammatory and anti-inflammatory cytokine secretion from keratinocytes. If recurrent CA-MRSA isolates suppress the TLR2 pathway via a secreted bacterial virulence factor(s), this could explain the ability of these isolates to result in more severe, and often recurrent, infections. In order to assess this possibility, we stimulated normal human epidermal keratinocytes (NHEKs) *in vitro* for 24 hours with three day bacterial culture supernatants from recurrent CA-MRSA or non-recurrent MSSA isolates. The downstream cytokine secretion from keratinocytes post exposure to either recurrent CA-MRSA or MSSA bacterial culture supernatants were measured by ELISA and compared to one another.

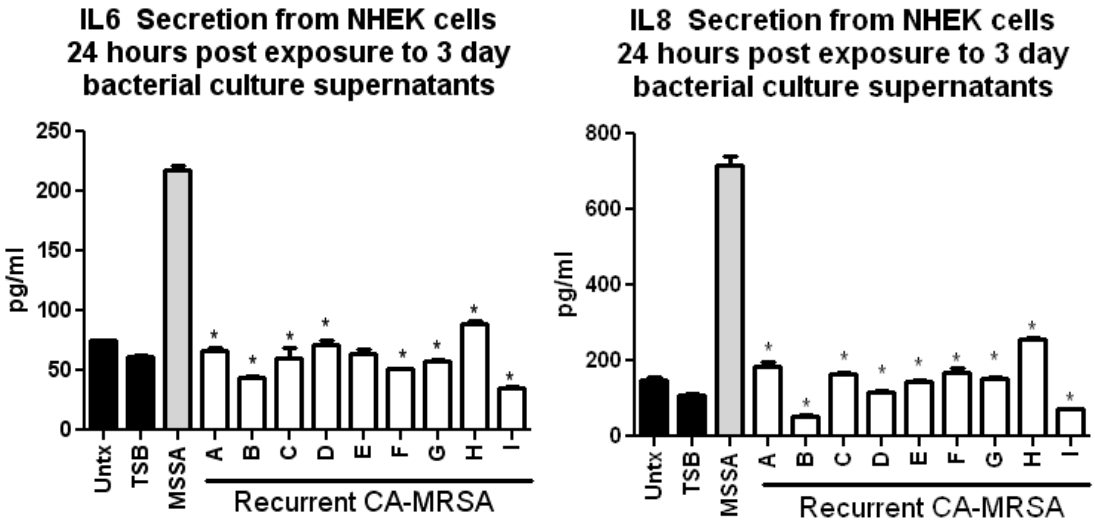
We found that recurrent CA-MRSA bacterial conditioned media exposure resulted in a significant decrease in secretion of IL6, IL8, IL10, and TNFα from keratinocytes (Figure 3). Keratinocytes exposed to recurrent CA-MRSA bacterial culture supernatants exhibited, on average, a fold decrease of 3.9, 6.0, 2.9, and 2.7 in IL6, IL8,

IL10, and TNFα secretion, respectively, relative to the cytokine secretion of cells exposed to MSSA bacterial culture supernatants.

Our samples included recurrent CA-MRSA isolates obtained from a variety of patients seen at the Loyola University Medical Center and an MSSA lab strain (Sa113). Table 1 lists the clinical information known about the patients that each of our isolates were obtained from.

Patient Initials	Recurrence Number	Date	Age	Sex	Source
2-15 RS	3	1/4/2012	56 yrs	M	Left foot
2-35 SB	5	3/20/2012	40 yrs	M	Right foot
2-37 FS	6	3/30/2012	70 yrs	M	Sinus
2-44 TB	2	4/15/2012	35 yrs	M	Ear
2-57 AA	1	12/9/2011	10 months	F	Left gluteal
2-45 AM	1	10/2/2011	17 yrs	M	Right leg
2-48 DM	2	5/14/2012	33 months	F	Groin
2-59 DD	1	10/31/2011	65 yrs	F	Abdominal Abscess
2-64 RW	2	6/16/2012	20 yrs	M	Pleural Fluid

Table 1: Clinical information about the recurrent CA-MRSA isolates used in preliminary bacterial culture media experiments. Each of the isolates in the above table was used in our bacterial culture supernatant exposure experiments outlined below.



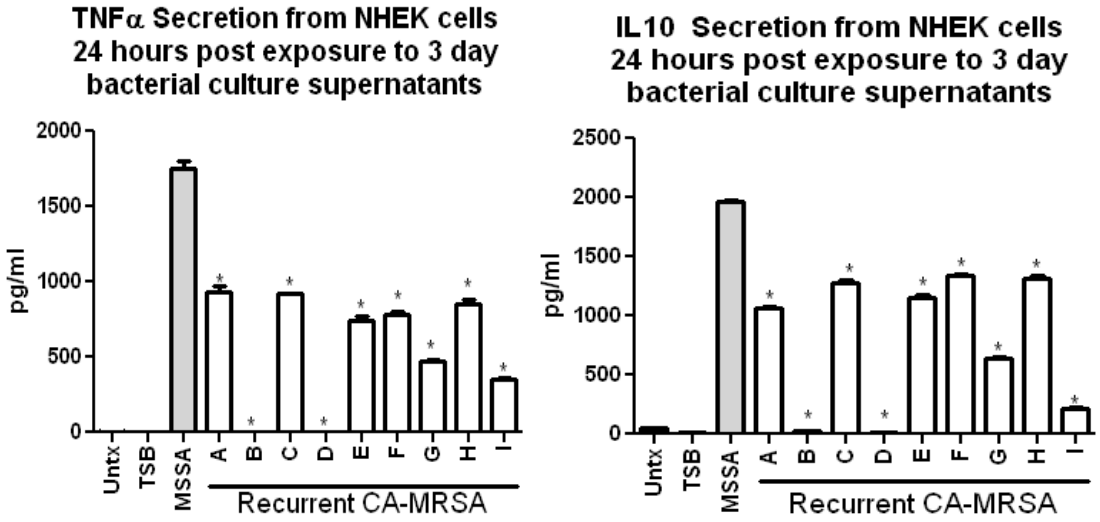


Figure 3: Pro-inflammatory and anti-inflammatory cytokine release by keratinocytes after treatment with CA-MRSA bacterial culture supernatants as shown by ELISA. NHEKs *in vitro* were treated with a 20% solution of bacterial culture supernatants from different recurrent CA-MRSA isolates from different patients (labeled with a letter corresponding to each patient) MSSA Sa113, TSB (negative control) or an untreated group for comparison. Keratinocytes treated with recurrent CA-MRSA bacterial culture supernatants exhibited decreased levels of secreted pro-inflammatory IL-6, IL-8 and TNFα as well as anti-inflammatory IL-10. *P<.0001 vs. MSSA treatment group after one way ANOVA with a Bonferroni multiple comparison post test.

HMGB1 secretion from NHEK cells post exposure to bacterial culture supernatants

TLR2 is expressed on keratinocytes and is a known receptor for HMGB1. HMGB1 binding to TLR2 results in subsequent release and production of pro-inflammatory and anti-inflammatory cytokines. We then asked if recurrent CA-MRSA isolates secrete a bacterial factor(s) that is able to suppress HMGB1 secretion from keratinocytes. Therefore, if recurrent CA-MRSA isolates are able to suppress TLR2 signaling through a secreted factor(s), we would expect the inflammatory effects of HMGB1 to be dampened as well. To answer this question, we again stimulated NHEKs *in vitro* for 24 hours with three day bacterial culture supernatants from recurrent CA-

MRSA or MSSA isolates. HMGB1 secretion from keratinocytes post exposure to either recurrent CA-MRSA or MSSA bacterial culture supernatants was measured by ELISA and compared to one another. We determined that recurrent CA-MRSA bacterial culture supernatant exposure resulted in a significant decrease in HMGB1 secretion from NHEKs (Figure 4). Keratinocytes exposed to recurrent CA-MRSA bacterial culture supernatants exhibited, on average, a fold decrease of 2.4 in HMGB1 secretion relative to the HMGB1 secretion of cells exposed to MSSA bacterial culture supernatants.

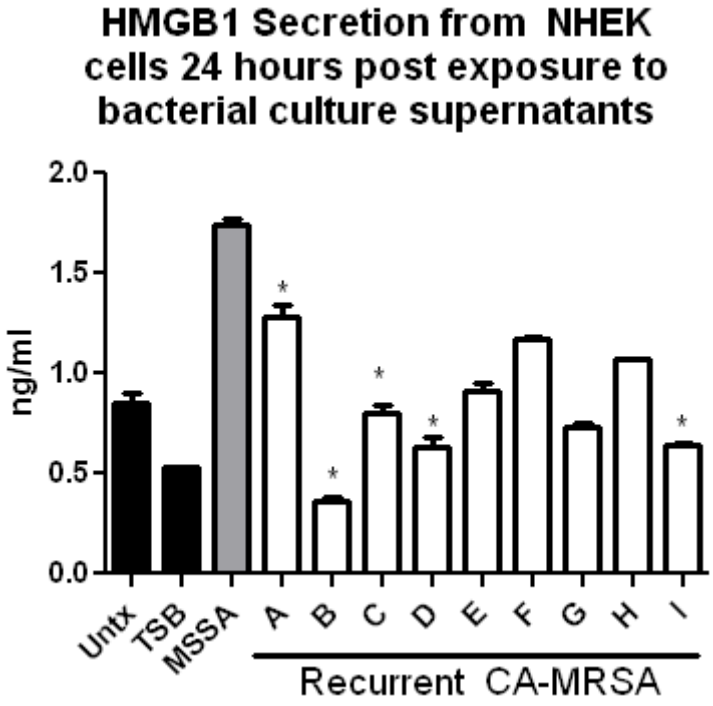


Figure 4: HMGB1 secretion measured from NHEKs exposed to MSSA or recurrent CA-MRSA bacterial culture supernatants for 24 hours measured by ELISA. Decreased levels of secreted HMGB1 were observed in supernatants from keratinocytes treated with recurrent CA-MRSA bacterial culture supernatants. HMGB1 levels were measured by ELISA and normalized to total protein.*P<.0001 vs. MSSA treatment group after one way ANOVA with Bonferroni's multiple comparison post test.

Cytokine secretion from HaCat cells post infection with live bacteria

Since we demonstrated that recurrent CA-MRSA bacterial culture supernatant exposure to keratinocytes was able to suppress cytokine secretion, we wanted to determine if cell-cell contact resulted in a similar decrease in downstream TLR2-mediated cytokine secretion. We then asked if live, recurrent CA-MRSA bacterial isolates were also able to suppress downstream TLR2 pro-inflammatory and anti-inflammatory cytokine production. In order to answer this question, we infected HaCat cells with live, recurrent CA-MRSA, non-recurrent CA-MRSA, or non-recurrent CA-MSSA bacterial isolates for 6 hours and compared relative levels of downstream cytokine secretion by ELISA.

Treatment of keratinocytes with recurrent CA-MRSA isolates resulted in a statistically non-significant decrease in pro-inflammatory TNFα and anti-inflammatory (IL10) secretion relative to cells treated with either non-recurrent CA-MSSA or non-recurrent CA-MRSA (Figure 5). Recurrent CA-MRSA isolate infection did result in a statistically significant decrease in pro-inflammatory IL8 secretion from keratinocytes post infection. A similar cytokine profile was observed with increased total cytokine levels at a 10 hour time point (data not shown). The clinical information of the isolates used in our live infection experiments are shown in Table 2.

Patient Initials	Recurrence Number	Date	Age	Sex	Source
FS	1	1/24/2011	69 yrs	M	Right sinus
	3	7/12/2011	70 yrs	M	Sinus
	6	3/30/2012	70 yrs	M	Sinus
TK	1	9/23/2011	50 yrs	M	Left leg
	4	11/12/2013	52 yrs	M	Left knee
CM	1	1/21/2011	84 yrs	F	Right foot
	4	8/13/2012	86 yrs	F	Right arm

AB	1	4/8/2011	40 yrs	F	Right hip
	4	1/29/2013	42 yrs	F	Right hip
SB	1	3/16/2011	39 yrs	M	Right big toe
	3	11/23/2011	39 yrs	M	Right big toe
	5	3/20/2012	40 yrs	M	Right foot
GF	1	11/4/2011	68 yrs	F	Left arm
	3	5/31/2012	69 yrs	F	Catheter drain site
PB	3	5/19/2011	63 yrs	F	Abdominal Abscess
	6	5/31/2013	65 yrs	F	Abdominal wall tissue

Table 2: Clinical information for the recurrent CA-MRSA isolates used in our live infection experiments. Each of the isolates in the above table was used to infect HaCat cells for 6 hours as outlined below. We selected an isolate from an earlier and a later recurrence within each of these randomly selected patients for these studies.

We then compared the effects of recurrent CA-MRSA isolates obtained from the first recurrence and those obtained from later recurrent infections of the same patient to determine if recurrence correlated with changes in cytokine secretion. Here, any infection that occurred at least three months after the initial infection was considered a recurrent infection. If exposure to isolates from subsequent CA-MRSA infections results in a decrease in pro-inflammatory and/or anti-inflammatory cytokine secretion from keratinocytes, this could suggest that these isolates are able to more effectively escape recognition by the TLR2 signaling pathway in the skin. We utilized the same live infection cell culture system as described above to assess cytokine secretion from keratinocytes 6 hours post infection by ELISA. Treatment of keratinocytes with CA-MRSA isolates from later recurrent infections revealed no statistically significant decrease in overall cytokine secretion (Figure 6).

We additionally looked at keratinocyte cytokine profiles post infection with CA-MRSA isolates from subsequent infections of the same patient. In some patients (Figure 7, [panels A-C), recurrent CA-MRSA isolates from later recurrences resulted in a non-

significant increase in overall IL10 secretion by keratinocytes post infection. In other patients (Figure 7, panels D-E), there was an observed non-significant decrease in IL10. Levels of TNFα and IL8 secretion from keratinocytes post infection were also not significantly different in subsequent infections of the same patient (Figure 7). Interestingly, five different non-recurrent CA-MSSA isolates consistently induced a similar cytokine secretion profile from keratinocytes post infection (Figure 7, panel F).

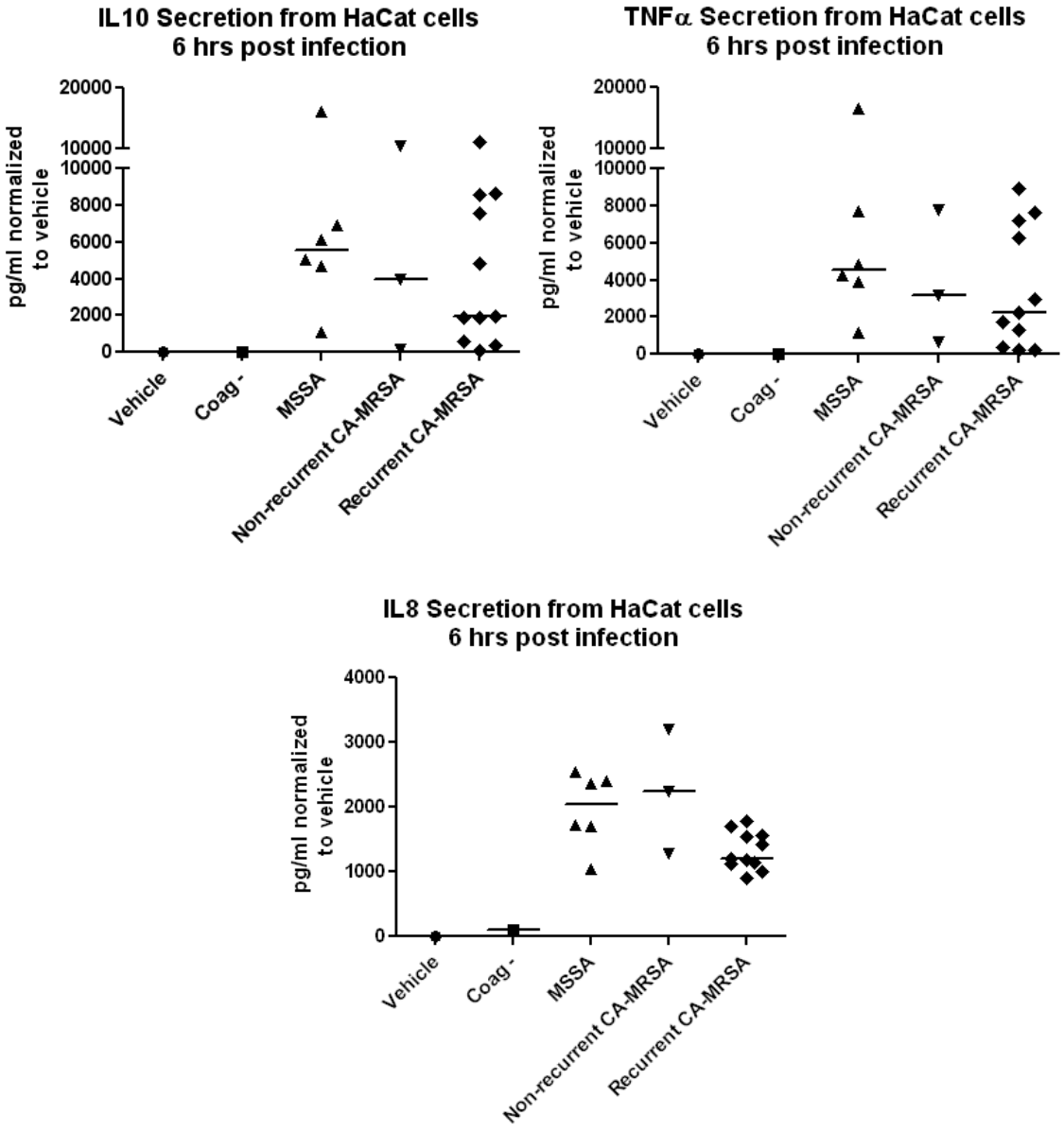


Figure 5: Relative pro-inflammatory (IL8 and TNFα) and anti-inflammatory (IL10) cytokine secretion levels from HaCat cells 6 hours post infection with either non-recurrent CA-MSSA clinical isolates, recurrent CA-MRSA clinical isolates, a vehicle control, or a coagulase negative *S. aureus* isolate (negative control), as measured by ELISA. There is a trend toward a decrease in cytokine secretion from cells treated with recurrent CA-MRSA vs. non-recurrent CA-MSSA isolates. Cytokine ELISA values of each isolates were averaged from three independent experiments; each dot indicates the average of the cytokine release of HaCat cells treated with a particular bacterial isolate. These data were analyzed by comparison of their medians due to the large variability and p values were obtained using a Mann Whitney U test as follows: IL10 p = 0.4510, TNFα p = 0.2478 and IL8 p = 0.0307. A statistically significant p value was designated as 0.05. Statistical significance was only noted in IL8 secretion, though a trend toward increased IL10 and TNFα secretion from cells treated with recurrent CA-MRSA isolates was identified. IL6 was also tested, but levels were very similar to the vehicle control in most samples and were therefore are not shown here.

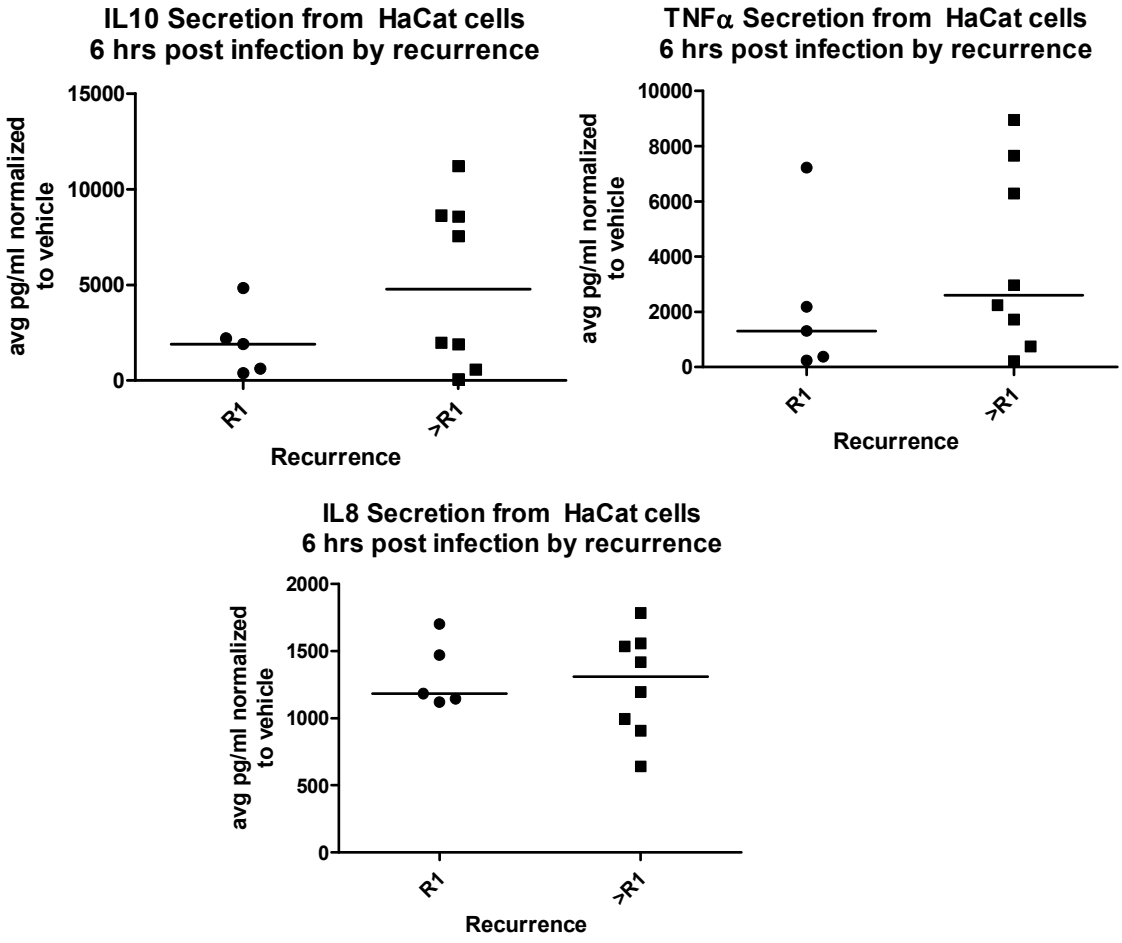
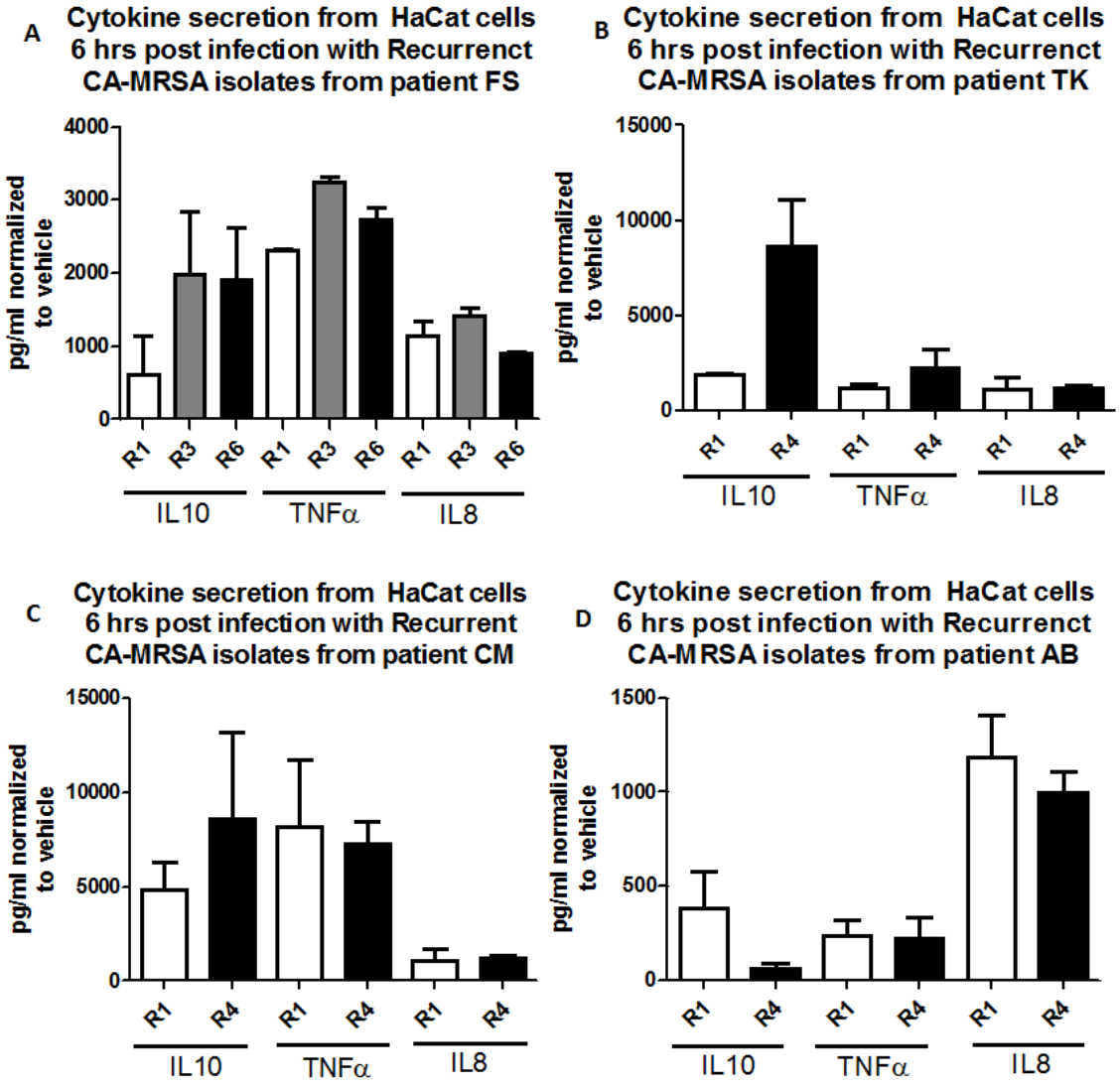


Figure 6: Relative pro-inflammatory (IL8 and TNFα) and anti-inflammatory (IL10) cytokine secretion levels from HaCat cells 6 hours post infection with recurrent CA-MRSA clinical isolates as measured by ELISA and grouped by recurrence. When the above cytokine data is organized based on the recurrence of the recurrent CA-MRSA isolate used to treat HaCat cells *in vitro*, overall patterns can be identified to predict changes in cytokine production that could be contributing to the ability of these isolates to cause recurrent infections. Cytokine ELISA values of each isolates were averaged from three independent experiments; each dot indicates the average of the cytokine release of HaCat cells treated with a particular recurrent CA-MRSA isolate. These data were then analyzed by comparison of their medians due to the large variability and p values were obtained using a Mann Whitney U test as follows: IL10 p = 0.4351, TNFα p = 0.3543 and IL8 p = 0.9433. No significance was noted, though a trend toward increased cytokine secretion during later recurrences was identified.



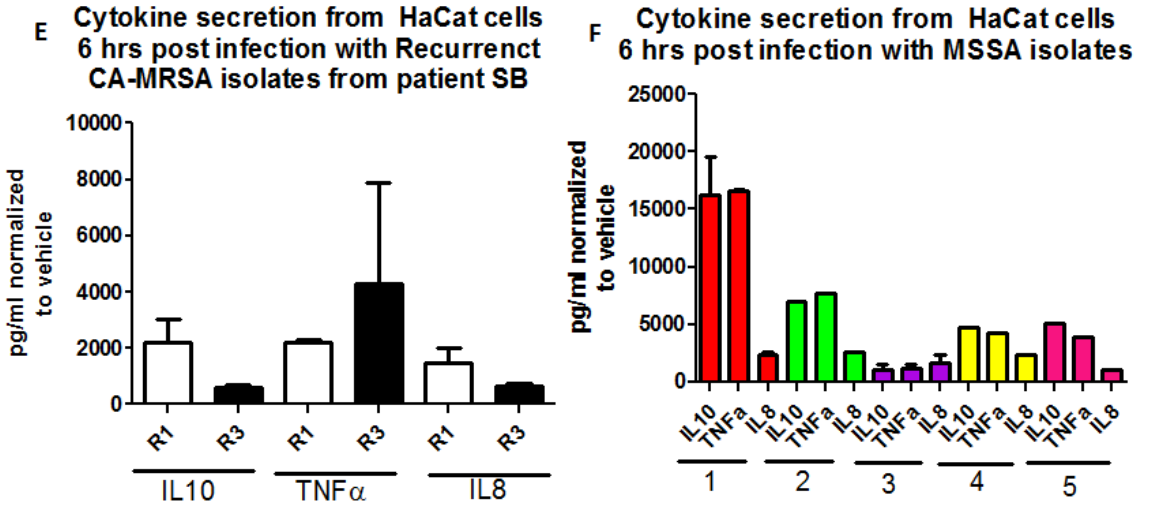
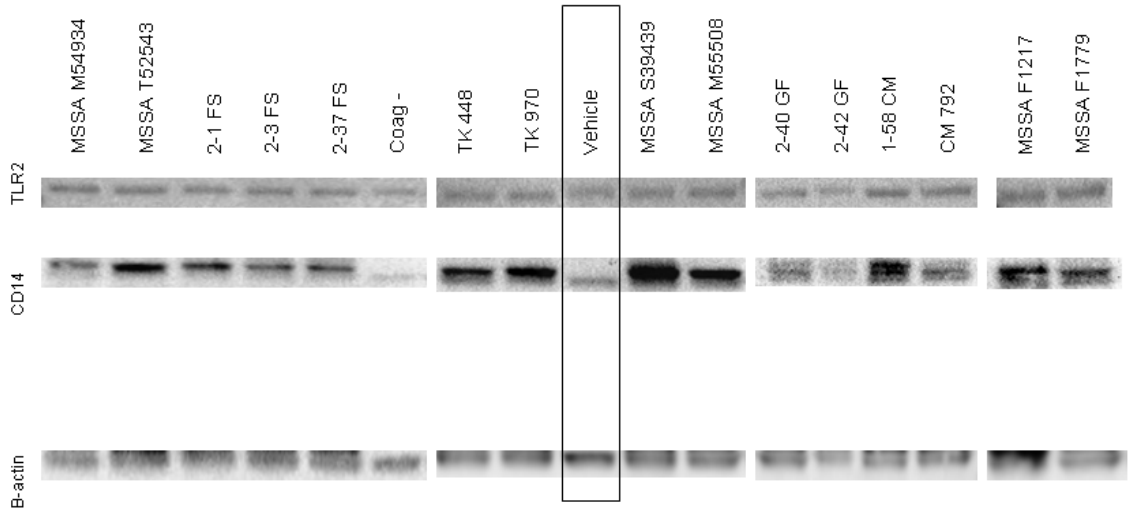


Figure 7: Relative pro-inflammatory (IL8 and TNFα) and anti-inflammatory (IL10) cytokine secretion levels from HaCat cells 6 hours post infection with subsequent CA-MRSA isolates from the same patient during recurrent infections (A-E) or with CA-MSSA isolates (F) as measured by ELISA. Differences in the cytokine profiles of cells treated with either non-recurrent CA-MSSA or subsequent recurrent CA-MRSA bacterial isolates from the same patient can be seen. Panels A-E show the cytokine profiles of cells treated with different recurrent CA-MRSA isolates from subsequent infections of the same patient. Panel F shows the cytokine profiles of the non-recurrent CA-MSSA isolates utilized in our studies.

Total protein expression of surface receptors in HaCat cells post infection with live bacteria

TLR2 is known to require recruitment of and interaction with one of its co-receptors, most commonly CD14, in order to recognize different lipopeptide structures on the surface of pathogens resulting in an innate immune response. Therefore, the next question that we asked was whether infection with recurrent CA-MRSA isolates results in a decrease in total protein levels of host surface receptors TLR2 and CD14 in keratinocytes. If recurrent CA-MRSA isolates decrease the total protein levels of TLR2 and/or CD14 in HaCat cells post infection, we would expect to see a decrease in pro-inflammatory (IL8 and TNFα) and anti-inflammatory (IL10) cytokine production in response to infection. In order to address this experimental question, we performed total

protein Western Blots on whole cell lysates from HaCat cells infected with non-recurrent CA-MSSA, non-recurrent CA-MRSA, or recurrent CA-MRSA isolates for 6 hours. These blots indicated relatively similar total levels of both TLR2 and CD14 in keratinocytes infected with non-recurrent CA-MSSA, non-recurrent CA-MRSA, and recurrent CA-MRSA isolates (Figure 8). The average densitometry values of each of the above groups in two independent experiments were determined and normalized to the loading control, β-actin (Figure 8). The medians of the recurrent CA-MRSA treated group were compared to the medians of the non-recurrent CA-MSSA and non-recurrent CA-MRSA groups, revealing a non-significant change in total TLR2 and CD14 protein expression at 6 hours post infection..



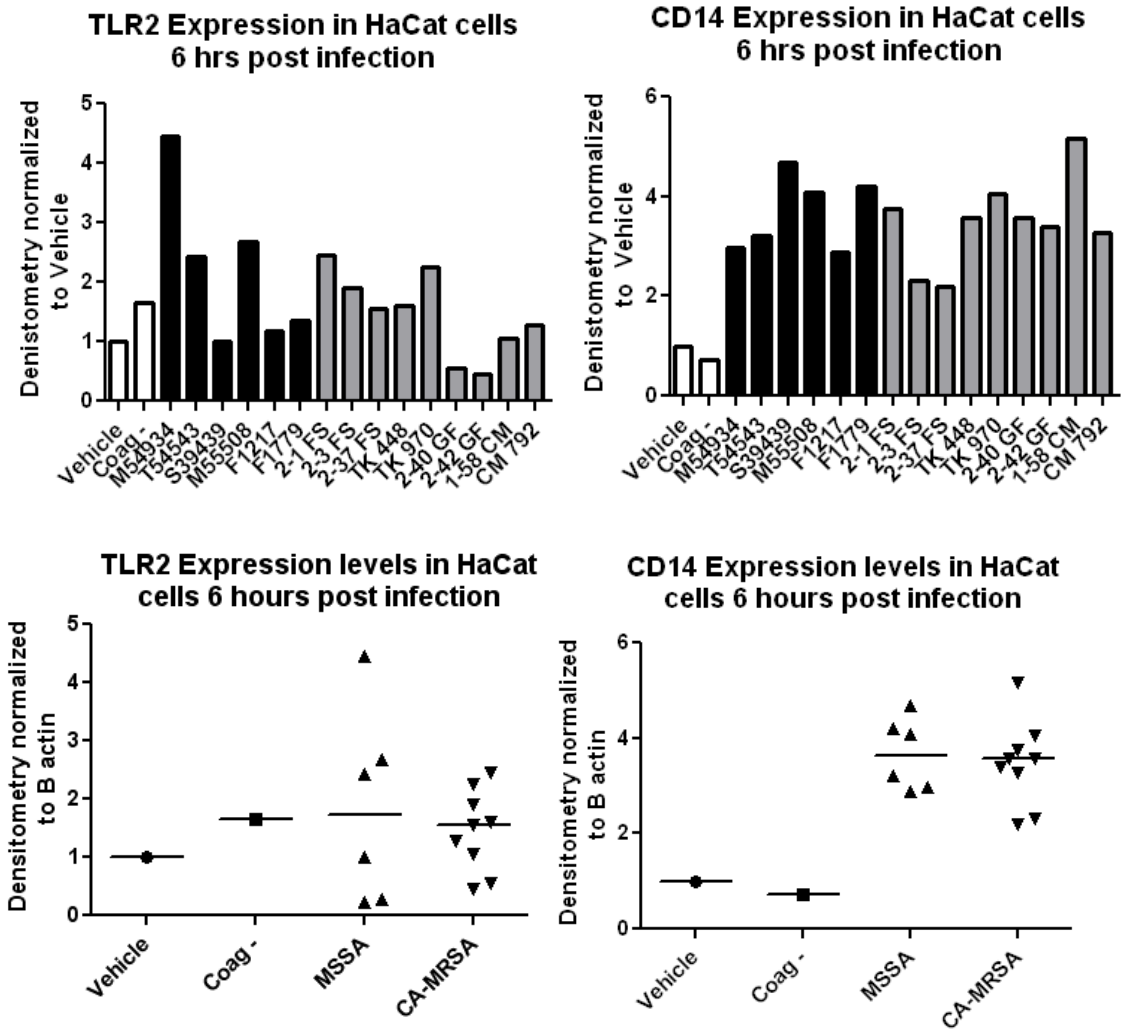


Figure 8: Western blot analysis of total HaCat cell lysates 6 hours post infection with MSSA isolates or recurrent CA-MRSA isolates with corresponding densitometry values normalized to B-actin suggesting relatively stable levels of TLR2 and CD14 post infection. Levels of total TLR2 remained relatively unchanged at this time point post infection. A dramatic increase in CD14 was also indicated in all MSSA (black bars) and recurrent CA-MRSA (gray bars) infected groups when compared to the negative control vehicle and coagulase-negative *S. aureus* treated groups as expected under infection conditions, but the levels between the infection groups were very similar and not statistically significant. These graphs show results from one experiment that was repeated again with similar results. P values were obtained using a Mann Whitney U test to compare MSSA and recurrent CA-MRSA treated groups; p values were as follows: TLR2 p=0.8438, CD14 p=0.7756. TLR1 was also imaged, but the band was very difficult to capture, but also appeared not to vary significantly between groups.

NFκB activation in HaCat cells post infection

Activation of the TLR2 signaling pathway ultimately leads to the phosphorylation and activation of the transcription factor, NFκB, which then leads to the upregulation of genes encoding for chemokines, cytokines, cell adhesion molecules, and AMPs (6). We next wanted to assess the ability of recurrent CA-MRSA isolates to inhibit NFκB activation via phosphorylation, which is a necessary event leading to downstream TLR2-mediated cytokine release. To answer this question, we carried out total phospho-NFκB Western Blots in order to compare the relative levels of NFκB activation between keratinocytes treated with recurrent CA-MRSA and non-recurrent CA-MRSA or CA-MSSA isolates. Since NFκB activation occurs very rapidly after infection, we assessed the total relative levels of NFκB and phospho-NFκB 15 minutes post infection with our bacterial isolates. If recurrent CA-MRSA isolates are able to inhibit the phosphorylation of NFκB, then we would expect to see a decrease in downstream pro-inflammatory and anti-inflammatory cytokine production. We found that keratinocytes infected with recurrent CA-MRSA isolates did not exhibit a significant change in the levels of total phospho-NFκB at 15 minutes post infection in our system (Figure 9).

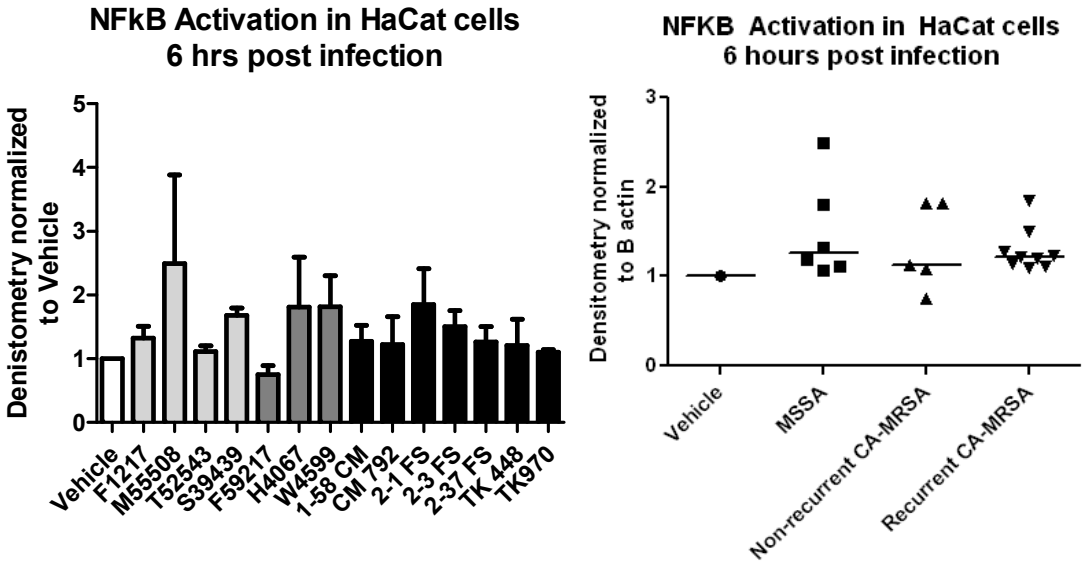


Figure 9: Western blot analysis of total HaCat cell lysates 15 minutes post infection with MSSA isolates or recurrent CA-MRSA isolates with corresponding densitometry values normalized to total NFκB. These data (using the mean of the normalized densitometry values of two experiments) suggest relatively similar levels of NFκB activation post infection. An overall increase in NFκB activation was observed when compared to the negative control vehicle and coagulase-negative *S. aureus* treated groups as expected under infection conditions, but these levels were relatively stable among treatment groups and not statistically significant. A p value was obtained using a Mann Whitney U test to compare CA-MSSA and recurrent CA-MRSA treated groups; p=0.8639.

HMGB1 secretion from HaCat cells post infection

HMGB1 is a danger associated molecular pattern (DAMP) that translocates from the nucleus to the extracellular milieu, upon TLR2 stimulation to elicit pro-inflammatory effects through NFκB activation. The next question that we investigated was if recurrent CA-MRSA isolates inhibit HMGB1 secretion from keratinocytes post infection as a mechanism to evade recognition by the host immune response. In order to answer this question, we carried out HMGB1 ELISAs on cell culture supernatants 6 hours post infection with recurrent CA-MRSA, non-recurrent CA-MRSA, and non-recurrent CA-

MSSA isolates. If recurrent CA-MRSA isolates inhibit the translocation of HMGB1 from the nucleus to the extracellular space, we would expect to see decreased secretion of HMGB1 from keratinocytes treated with recurrent CA-MRSA isolates. Infection of keratinocytes with recurrent CA-MRSA isolates did not exhibit a significant decrease in HMGB1 secretion 6 hours post infection in our system (Figure 10).

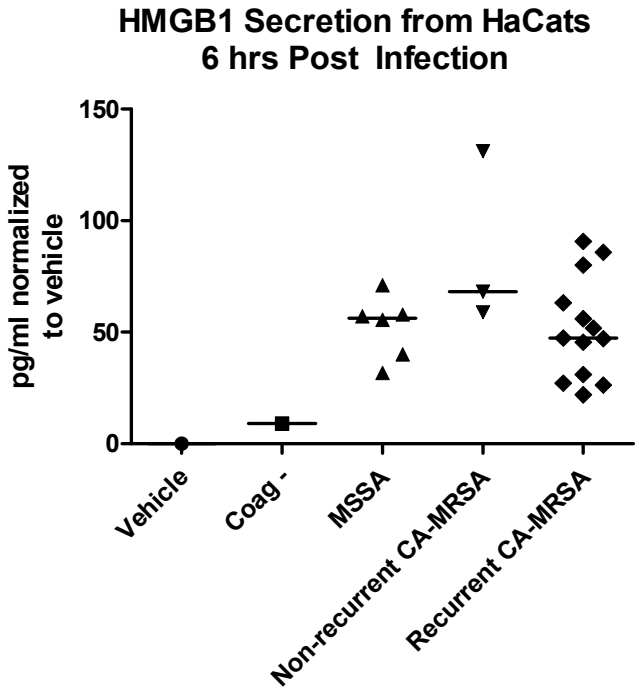


Figure 10: Relative HMGB1 secretion from HaCat cells 6 hours post infection with either non-recurrent CA-MSSA clinical isolates, recurrent CA-MRSA clinical isolates, a vehicle control, or a coagulase negative *S. aureus* isolate (negative control), as measured by ELISA. Stimulation of HaCat cells for 6 hours with live bacterial isolates resulted in no significant difference in HMGB1 secretion between CA-MSSA and recurrent CA-MRSA treated groups, indicating that recurrent CA-MRSA bacteria are probably not affecting the secretion of this protein as a mechanism to cause decreased inflammatory response and immune avoidance during infection. A p value were obtained using a Mann Whitney U test to compare MSSA and recurrent CA-MRSA treated groups; p=0.6931.

HMGB1 localization in HaCat cells post infection

The function of HMGB1 is dependent on its location within the cell. Under normal conditions HMGB1 acts as a DNA-binding protein and transcription factor in the nucleus (1). Under inflammatory conditions, such as IL6 and TNFα production, this protein can be translocated from the nucleus to the cytoplasm. In the cytoplasm it is packaged into vesicles prior to its secretion into the extracellular space, where it can then bind TLR2 (1) in order to amplify the inflammatory response. We then asked if recurrent CA-MRSA isolates inhibit the translocation of HMGB1 from the nucleus to the cytoplasm, or from the cytoplasm into the extracellular space in keratinocytes post infection. In order to answer this question, we carried out ICC assays on keratinocytes 6 hours post infection with either recurrent CA-MRSA or non-recurrent CA-MSSA isolates in order to compare relative levels of HMGB1 localization by utilizing a fluorescently conjugated HMGB1 antibody. Similarly, if recurrent CA-MRSA isolates inhibit the translocation of HMGB1 from the cytoplasm to the extracellular space, we would expect to see increased levels of cytoplasmic HMGB1 in keratinocytes infected with these isolates. Unfortunately we were not able to address this question using this method due to technical complications. Here, the fluorescent signal due to the bacteria binding the fluorescently conjugated secondary antibodies was very high and it was difficult to determine localization of HMGB1 in the cells treated with the bacteria groups (Figure 11).

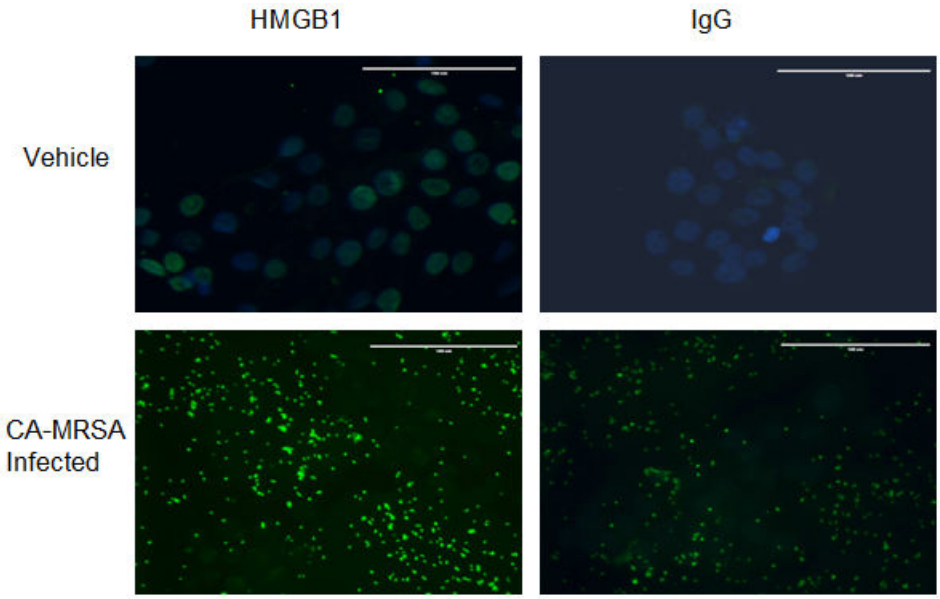


Figure 11: HMGB1 localization via ICC with vehicle treated and CA-MRSA treated groups. Here, ICC was carried out using an HMGB1 specific antibody or IgG isotype control at 6 hours post infection. The vehicle treated cells show high levels of HMGB1 in the nucleus as expected under normal conditions and no staining in the IgG control indicating no non-specific binding of the antibody. The CA-MRSA treated group exhibits high levels of punctate staining that is localized to the bacteria on the slide and is also present a high degree in the IgG control. This FITC signal is so high in the CA-MRSA infected cells that I am not able to visualize the cells in the vehicle and infected groups under the same microscope conditions, so valid comparisons can not be made between groups at this time. Additional troubleshooting will be needed in the future to answer our experimental questions regarding localization and co-localization in infected cells with immunoassays due to the presence of protein A as discussed previously.

CHAPTER FIVE

DISCUSSION

Taken together, our data provide some insight into the inflammatory effects of different clinical recurrent CA-MRSA and non-recurrent MSSA isolates on keratinocytes *in vitro*. It is important to identify differences in the immune response against these pathogens in order to understand why CA-MRSA isolates possess enhanced virulence and are often able to cause recurrent infections in otherwise healthy individuals. If particular mechanisms underlying the interactions between the bacteria and the host immune system are identified, these pathways could serve as targets for improved treatment of CA-MRSA infections, particularly those that result in recurrence.

In all of our analyses we compared the effects of recurrent CA-MRSA isolates on keratinocytes to the effects of non-recurrent CA-MSSA isolates under the same experimental conditions. Non-recurrent CA-MSSA isolates were the appropriate comparison to recurrent CA-MRSA isolates because of their evolutionary background. CA-MRSA is thought to have emerged from CA-MSSA, as opposed to HA-MRSA, due to the sizes of their SCCmec cassettes (24). Therefore, making a comparison between recurrent CA-MRSA and non-recurrent HA-MRSA would be potentially misleading in our mechanistic analyses but could be pursued in later phases of this project. Additionally, in later experiments, we included a few non-recurrent CA-MRSA bacterial isolates in our analysis and determined that there appears to be similar variability and

patterns in cells treated with these isolates. The sample size for these isolates was very small with only three isolates and could be pursued on a larger scale in the future.

Exposure of keratinocytes to recurrent CA-MRSA bacterial culture supernatants

TLR2 is known to be the main recognition receptor of *S. aureus*, therefore, it is reasonable to speculate that CA-MRSA is able to cause recurrent infections by circumventing TLR2 recognition and signaling. Differences are known to exist between the production of secreted virulence factors, such as superantigens, by CA-MRSA and CA-MSSA (26). In our initial preliminary experiments, we grew up different recurrent, clinical CA-MRSA isolates and an MSSA (Sa113) isolate in culture for three days prior to collecting the culture supernatants. These supernatants included any factors that the bacteria secreted in the three day time frame as well as any shed cell wall components. We demonstrated that exposure of NHEKs to recurrent CA-MRSA bacterial culture supernatants resulted in a significant decrease in secretion of pro-inflammatory (IL8, IL6 and TNFα) and anti-inflammatory (IL10) cytokines post infection. Additionally, we saw an increase in total levels of HMGB1 and a decrease in the secretion of HMGB1. These findings suggested that a secreted or shed cell wall component(s) produced by recurrent CA-MRSA isolates was able to decrease the innate immune response in the skin to a greater degree than the MSSA isolate tested. These effects could have been mediated by a cell wall component(s) such as lipoteichoic acid, or by a secreted virulence factor(s) produced by the bacteria at some point during the three day time course.

One possible explanation for the observed decrease in presumed TLR2-mediated cytokine secretion from keratinocytes after exposure to recurrent CA-MRSA bacterial culture supernatants, is the secreted *S. aureus* virulence factor staphylococcal superantigen-like protein 3 (SSL3) (36). This family of staphylococcal superantigen-like proteins is becoming of increasing interest due to their described ability to mediate immune evasion. SSL3 is able to specifically bind the extracellular domain of TLR2 on neutrophils and monocytes in mice leading to a reduction of downstream cytokine production (36). Since keratinocytes are also known to express TLR2, we hypothesize that this virulence factor is upregulated by recurrent CA-MRSA isolates. This would also explain why we were unable to reproduce the decrease in TLR2-mediated cytokine secretion by keratinocytes in our live infection model as the bacteria were washed prior to infection, removing any secreted factors that they may have produced. Additionally, the six hour infection that was utilized would possibly not have been enough time to produce enough of this virulence factor to cause a noticeable effect in downstream TLR2 signaling in our system.

It has also been shown that bacterial culture supernatants of the *Staphylococcus epidermidis*, were able to activate TLR2 signaling resulting in an increase in the production of AMPs and increased defense against infection (74). This study further highlights the possibility that secreted virulence factors, even of commensal species, are able to affect host signaling pathways. It also showcases a limitation of our cell culture model. In our model, we do not have the effects of other bacteria, including commensals, which could also be playing a role in the establishment of recurrent infection.

Heterogeneity in the effects of live infection of keratinocytes with recurrent CA-MRSA isolates in vitro

We speculated that cell-cell contact between recurrent CA-MRSA isolates and keratinocytes may also be involved in the observed suppression of the inflammatory response, acting as a possible contributing factor in the pathogenesis of recurrent CA-MRSA infection. An *in vitro* model using the immortalized HaCat cell line was utilized to allow us to asses the ability of recurrent CA-MRSA isolates to suppress TLR2-mediated pro-inflammatory cytokine secretion from keratinocytes during infection. Our data suggested that there was a large degree of heterogeneity in downstream TLR2 inflammatory events in cells treated with recurrent CA-MRSA and non-recurrent CA-MSSA isolates in keratinocytes.

The observed decreases in relative levels of cytokine secretion, surface receptor expression, or NFκB activation were likely not statistically significant due to the large biological heterogeneity in the inflammatory response post infection between individual isolates. We knew very little clinical information about the patients that our isolates were obtained from. There are a number of host and environmental factors that could have affected the bacteria isolated. These include the severity of the infection at the time of bacterial isolation, if the patient was on a current or recent antibiotic regimen, or even the genetic dispositions or other comorbidities of these patients. All of these factors have the potential to influence the environment in which the bacteria are adapting and could affect their overall physiology and pathogenicity. Additionally, the isolates that we investigated were not strain typed so it was unclear if the same bacterial strain is the one that is

responsible for recurrent infections or if they are different strains. This is something that should be investigated in the future to better understand if recurrent infections are due to the adaptation of the bacteria or if they are due to host factors.

Another recent study utilized human embryonic kidney (HEK) cells transfected with TLR2 activity in order to assess the ability of different clinical *S. aureus* isolates to stimulate TLR2. Here, IL8 cytokine production was used as a readout of TLR2 activation 24 hours post infection (46). This experimental set up was similar to ours and these investigators also identified a large degree of heterogeneity in the TLR2 activity exhibited by different *S. aureus* isolates as we observed in our studies. These investigators also compared TLR2 activation post infection of HEK cells with isolates obtained from patients with either cystic fibrosis or invasive disease. They found no correlation between the origin of the isolate and the TLR2 activity (46). Our findings extend these observations in that we observed a similar pattern in keratinocytes stimulated with clinical non-recurrent CA-MSSA or recurrent CA-MRSA isolates for 6 hours. Additionally, these investigators looked at a variety of cellular processes to discover which one(s) were responsible for these changes in TLR2 activity. They discovered that this activity appears to be due to a variety of factors including proliferative activity, capsule formation, protein synthesis and cell wall factors (46).

HMGB1 function during recurrent CA-MRSA infection

It is known that mammalian cells do not produce new pools of HMGB1 until about 16 hours post stimulation (27). The HMGB1 utilized and secreted by the cell prior

to this time point is from a preformed nuclear store of this protein (27). Therefore, we would not expect to see any changes in the total HMGB1 levels, which we observed in our system via Western Blot (data not shown). We were unfortunately limited with our cell culture model in terms of the amount of time that we could expose our cells to the live bacteria in culture prior to killing the cells. Importantly, we were still able to assess the secretion of HMGB1 into the extracellular space post infection. This allowed us to determine that recurrent CA-MRSA isolates do not suppress the secretion of HMGB1 from keratinocytes post infection relative to the levels of HMGB1 secretion from non-recurrent CA-MSSA treated groups. This indicated that there was not a variation in the amount of HMGB1 in the extracellular space available to interact with TLR2 at the cell surface that could stimulate the TLR2 signaling pathway. Since recurrent CA-MRSA isolates did not result in a significant decrease in pro and anti-inflammatory cytokine secretion from keratinocytes post live infection, we did not carry out the initially proposed addition of exogenous HMGB1 experiments. Therefore, HMGB1 secretion was not affected by recurrent CA-MRSA cell surface virulence factors leading to recurrent infections under our experimental conditions. Although, HMGB1 secretion was decreased after exposure to secreted factors from recurrent CA-MRSA isolates. Due to these findings, it is possible that a secreted virulence factor(s), as opposed to a cell surface virulence factor, may be responsible for the ability of these bacteria to cause recurrent infections in otherwise healthy individuals.

Limitations of our in vitro keratinocyte infection model

There were a number of limitations in our live infection cell culture model. We were limited in the amount of time that we could expose the cells to the live bacteria. We chose the 6 hour time point after performing an LDH cytotoxicity assay (data not shown). Cell death was assessed at different time points 2-24 hours post infection and revealed that after 8 hours, there was increased cell death and cellular morphology changes in keratinocytes infected with live bacteria. We also based our time point on a study looking at the kinetics of cytokine production after LPS stimulation of human whole blood (25). This study determined that TNFα protein production peaked about 4-6 hours post infection before stabilizing and that IL8 protein increased initially up to about 6 to 12 hours post stimulation (25). Though this was a very different system than ours, it was a helpful starting point to determine when we would be able to see changes in protein expression in our cells post infection. It has also been found that peak cytokine responses post infection of cells with Gram positive bacteria occurs 50 to 75 hours after the challenge, as opposed to 1 to 5 hours after a Gram negative challenge (57). This means that we may have needed to expose our cells to the live bacteria for much longer than 6 hours to see changes in the response of the keratinocytes post infection. A possible way to address this limitation in the future could be by using heat killed bacteria so that their cell wall components would still be exposed to the cells. Here, the bacteria would not be dividing and invading cells so the cytokine profiles could be examined for longer periods of time. The problem with this method is that if the bacteria are not growing and dividing,

then infection conditions are not being replicated which could lead to a different immune response by the host cells.

Our system was also limited to looking at the effects of CA-MRSA isolates on keratinocytes alone and vice versa. It is also likely that there are other confounding factors that my influence these interactions *in vivo* and with the contributions of other immune cells and cellular processes. Additionally, the cytokine secretion profiles that we investigated would directly and indirectly affect the recruitment and activity of other immune cell types. For example, IL10 and TNFα have been shown to play bilateral roles during injury and infection (11). IL10 is an anti-inflammatory cytokine that skews the immune response toward a Th2 response by promoting B cell survival and proliferation. TNFα is a pro-inflammatory cytokine that is known to activate macrophages. And finally, IL8 is another pro-inflammatory cytokine that is produced in response to infection and plays a role in neutrophil activation and recruitment to sites of infection. Therefore, the production of these cytokines is only one aspect of the host immune response to infection that could be affected by recurrent CA-MRSA virulence factors.

The challenge of live bacterial infection in vitro

Working with live bacteria posed a number of problems in regards to our assays and much troubleshooting was done to try to overcome these obstacles. It is important to note some of these challenges in order to address them more effectively in future studies in the field.

The largest problem was trying to find ways to overcome the antibody binding activity of the *S. aureus* virulence factor, Protein A. Since we infected cells with *S. aureus* bacteria for 6 hours prior to collection, the cells contained bacteria that were internalized during the incubation period. Protein A is a virulence factor secreted by *S. aureus* that binds the F_c portion of immunoglobulin molecules to prevent opsonization and antibody recognition of the pathogen (6). This protein was a problem when trying to carry out the initially proposed co-immunoprecipitation experiments to assess for protein-protein interactions between TLR2 and its co-receptors. The protein A that was contained in the bacteria used in the assay was presumably leading to nonspecific binding of the antibodies utilized. We saw heavy bands appearing around 50 kD and 25 kD (data not shown) which we presume were showing up where the antibodies were recognizing the heavy and light chains of the TLR2 antibody used in the pull down. This problem would have prevented us from visualizing CD14 and HMGB1 via co-immunoprecipitation due to their molecular weights that are close to that of the heavy chain (~50 kD).

To solve this problem, we tried to utilize protein G beads (as opposed to protein A/G beads) for the Co-IP experiments but we were still detecting the problematic bands as encountered previously. The likely reason for this was that after complete cell lysis, protein A was present in high amounts and able to bind the F_c regions of any IgG molecules, including the Rabbit IgG used in the pre-clear step, the primary antibodies, and the secondary antibodies. During the pre-clearance step we did add a large amount of normal rabbit IgG to remove any non-specific binding and to allow for the removal of protein A from our samples. It is likely that this did not remove all protein A from the

sample. The protein A that remained would then be able to competitively bind with the primary antibody and lead to decreased efficiency of the pull down of the protein of interest. This additionally results in the pull down of protein A and therefore, a large amount of the primary antibody along with it, which are then visualized on the Western Blot downstream. We used an easy blot secondary antibody (Genetex, Ca. No. GTX221666-01) that only recognizes non-reduced antibodies and should not detect bands due to the heavy and light chains. Unfortunately, the protein A can also bind the secondary antibody non-specifically resulting in visualization of the IgG heavy and light chain bands by Western blot. This was also likely the reason why using a primary antibody from a different species also resulted in bands where we would expect the heavy and light chains, since protein A does not differentiate between species of IgG. This problem is likely not encountered in straight Western Blot assays, because the amount of protein A with all of the other proteins present in the sample is minute, while in the Co-IP protocol, these lysates are concentrated.

With these considerations and experiences in mind, and in the interest of time, we decided to use an ICC protocol that was well established in the lab to look at the co-localization of TLR2 and its co-receptors. This method does not give a direct indication of protein-protein interactions but instead provides strong evidence for co-localization between TLR2 and its co-receptors, which is required prior to TLR2 activation. Unfortunately, this assay posed its own challenges as well. In the ICC protocol, the cells that were infected with the bacteria were binding to the fluorescent antibodies and producing non-specific florescent staining (Figure 11). This non-specific staining was

slightly improved by changing the blocking reagent to a solution of 10% heat inactivated FBS, as opposed to the superblock reagent (Scytek Laboratories, Cat. No. AAA125), but this did not solve the problem. This was likely due to the large amounts of bacteria that remained on the slides even after additional wash steps. Increasing the FBS concentration and/or blocking time (>30 minutes) may further solve this problem, but it should be noted that we are not aware of any higher concentrations of FBS being used in assays such as these. It might be beneficial to test varying concentrations of FBS on the efficiency of the block while also being careful not to over saturate the system with antibodies. Also, a number of our antibodies that we purchased for use in these experiments did not work well in HaCat cells under out experimental conditions with either acetone or paraformaldehyde fixation. We could not visualize any staining by some of these antibodies on our Evos microscope. We did try to use deconvolution microscopy to determine if the signal produced by these antibodies was just very low due to the conditions in my system, or if the antibodies were not binding appropriately to the cells. This method utilizes a number of mathematical algorithms to analyze images from different focal planes and combines them to form a clearer three dimensional image (67). After doing this, we were still unable to see any florescent signal except for the signal that was due to the bacteria binding the secondary antibody (data not shown). The co-localization of CD14 and TLR2 in human monocytes after LTA exposure has been demonstrated successfully with the use of confocal microscopy (75). Additionally, TLR2 has been imaged by ICC successfully in murine keratinocytes using confocal microscopy (76). The success of these experiments indicate that trying different imaging techniques

may also be required to assess co-localization between TLR2 and its co-receptors and should be further pursued in the future.

Future directions

This project was the first to try to answer the question of how CA-MRSA isolates are able to cause recurrent infections. Therefore, there are still a number of unanswered questions and future directions that could be pursued.

The co-localization of TLR2 and its co-receptors is still a very important question that should be addressed, but under our experimental conditions, it was difficult to overcome the problem of protein A activity in our samples. Therefore, the troubleshooting mentioned above should be pursued in order to assess these interactions. Additionally, it has come to our attention that HaCat cells do not express TLR6 (28), while primary skin cells do, so determining the interaction of TLR2 with this co-receptor was removed from our experimental methods. HaCat cells are an immortalized cell line and have variable expression of particular TLRs when compared to NHEKs and the human epidermis (28). Therefore, in the future, these processes should be further investigated in an *in vitro* model using NHEKs or an *ex vivo* skin infection model using human epidermis samples to further establish the clinical relevance of our findings.

We have been unable to successfully separate nuclear and cytosolic extracts from HaCat cells. This is possibly due to the large size and high protein content of these cells (at least 5x more protein content than primary cells). We attempted to grow the cells up in increasingly smaller cell culture dishes with increasing efficiency, but have still not

been able to completely separate these extracts. Due to this, we were only able to assess the total levels of phospho-NFκB, NFκB, and HMGB1 and were not able to look at the cellular compartmentalization of these molecules. Looking at these total levels still provided us with valuable information on if recurrent CA-MRSA isolates could decrease the levels of NFκB activation as hypothesized. An alternative approach to look at localization of NFκB in the future with total protein lysates, could be to probe total protein Western Blots with an antibody against IκB, the inhibitor protein that keeps NFκB in the cytoplasm. Upon phosphorylation of this protein, a nuclear localization signal on NFκB is exposed and it is transported into the nucleus and the IκB protein is tagged for degradation by the proteosome. Looking at the levels of phospho-IκB, as compared to total levels of IκB, in total protein lysates could help to determine the amount of nuclear NFκB indirectly. Though, based on our results, it does not appear that NFκB translocation is effected in our assay due to the relatively stable levels of total P-NFκB present, which requires NFκB to get into the nucleus prior to phosphorylation and activation. Additionally, troubleshooting experiments should be continued to determine the best protocol to separate the nuclear and cytosolic extracts of these samples in order to directly determine the localization of these molecules post infection in keratinocytes.

In our ICC experiments, though the cells stained well in the vehicle control, it was difficult to analyze the infection and vehicle groups with the same microscope settings due to the differences in light intensity. Even with appropriate antibodies this would still be a problem with any ICC experiments that utilize our experimental conditions with live bacteria. Potentially in the future, the concentration of bacteria could be decreased to

improve this problem. Additionally, confocal microscopy may be able to be used with more appropriate antibodies to solve this problem. This method allows you focus more closely and clearly on a smaller area of your slide that might allow for the avoidance of the signal due to the bacteria binding the antibodies in the assay (80). Of note, it was difficult to separate what was true staining of the cells and what staining was caused by the bacteria in this assay. It may be useful to use a structural stain as well for these assays to be able to visualize the boundaries of the cells instead of just the nucleus.

In the interest of time, I was not able to perform the propposed CHiP assays. In a future project, the CHiP assays would give a good indication of possible changes at the gene level, as opposed to the protein level where the rest of our experiments are focused. Of note, since the binding of HMGB1 is known to be non-specific and low affinity, it may be difficult to capture this binding interaction in a ChIP assay. Another possible complication is the time frame of these experiments. We have proposed to look at a 6 hour time point, but it is also possible that these transcriptional changes could occur at various time points during infection.

And finally, we believe that it would also be beneficial to further investigate the effects of the secreted factors on TLR2-mediated cytokine secretion from keratinocytes post infection as that is where we were able to see the most notable difference between non-recurrent CA-MSSA and recurrent CA-MRSA treated groups. If these studies are to be carried out, they should be done with additional MSSA isolates to make sure that the findings still hold true when using clinical CA-MSSA isolates, as opposed to a lab MSSA strain (Sa113). Additionally, these studies could be extended to a larger number of

samples. Due to the large variability within our samples, this would be a good idea in order to determine the clinical relevance of these findings. It would also be beneficial to use a TLR2 neutralizing antibody to determine if these effects are in fact mediated by TLR2, as opposed to some other recognition receptor in keratinocytes such as RAGE or TLR4. Due to the above mentioned cell culture model limitations, it is very important to develop *ex vivo* and *in vitro* models to further these findings. All of these experiments would allow us to better understand the interactions between recurrent CA-MRSA isolates and keratinocytes and would potentially allow for creation of better immunomodulatory treatments for recurrent CA-MRSA infections in the clinic.

Summary and conclusions

In conclusion, the goal of this project was to better understand how recurrent CA-MRSA bacterial isolates are able to establish recurrent infections in otherwise healthy individuals. It was hypothesized that this was possible due to the production of a cell-surface virulence factor by these isolates that circumvented the TLR2 recognition signaling pathway in keratinocytes. We found that exposure of keratinocytes to bacterial culture supernatants from recurrent CA-MRSA isolates resulted in significantly decreased secretion of pro-inflammatory (IL6 and TNFα) and anti-inflammatory (IL10) cytokines. We further demonstrated that live keratinocyte infection with recurrent CA-MRSA isolates did not significantly alter the production of these cytokines. We also identified no significant difference in cytokine secretion from keratinocytes treated with CA-MRSA isolates from subsequent infections of the same patient. Finally, we

determined that recurrent CA-MRSA isolates did not decrease NFκB activation, total TLR2 or CD14 protein levels, or HMGB1 secretion under our experimental conditions.

Our hypothesis suggesting that recurrent CA-MRSA cell surface virulence factors were responsible for the decreased recognition by TLR2 host receptors proved to be incorrect. Our findings suggest that a secreted virulence factor(s) may responsible for the observed suppression of the inflammatory responses in keratinocytes post infection with recurrent CA-MRSA isolates. These findings have important implications in the clinic and in how physicians currently manage recurrent CA-MRSA infection.

The next steps should be to further determine the involvement of particular secreted virulence factor(s) in order to potentially use it as a target for further immunotherapies of recurrent CA-MRSA. As previously mentioned, a promising starting point to these analyses is the further investigation of the SSL3 protein and its expression during recurrent CA-MRSA infections. This protein was discovered relatively recently so there are no commercial antibodies or PCR probes against this antigen. This means that future work could be done in developing these tools for use in immunoassays in order to determine if SSL3 may be playing a role in the ability of CA-MRSA to cause recurrent infections. Based on our findings, we hypothesize that recurrent CA-MRSA isolates upregulate SSL3 expression resulting in a decrease in TLR2-mediated cytokine secretion from keratinocytes and therefore, decreased recognition of the pathogen in the skin.

REFERENCES

1. Hari, Aswin, et al. "Toll-like Receptors: Role in Dermatological Disease." *Mediators of Inflammation.* Vol. 2010 (2010), article ID 437246: 16 pages.

2. Ouhara, Kazuhisa, et al. "Increased resistance to cationic antimicrobial peptide LL-37 in Methicillin-resistant strains of *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy.* Vol. 61 (2008): 1266-1269.

3. Komatsuzawa, Hitoshi, et al. "Innate defenses against methicillin-resistant *Staphylococcus aureus* (MRSA) infection." *Journal of Pathology*. Vol. 208 (2006): 249-260.

4. Liu, George Y. "Molecular Pathogenesis of *Staphylococcus aureus* Infection." *Pediatric Research.* Vol. 65, No. 5, part 2 (2009): 71R-77R.

5. Hoffmann, Elke, et al. "Multiple control of interleukin-8 gene expression." *Journal of Leukocyte Biology*. Vol. 72, No. 5 (2002): 847-855.

6. Nizet, Victor. "Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets." *Journal of Allergy and Clinical Immunology*. Vol. 120(1) (2007): 13-22.

7. Graves, Shawna F. and Scott D. Kobayashi. "Community-associated methicillin-resistant *Staphylococcus aureus* immune evasion and virulence." *Journal of Molecular Medicine*. Vol. 88, No. 2 (2010): 109-114.

8. Takeuchi, Osamu, et al. "Cutting Edge: TLR2-Deficient and MyD88-Deficient Mice are Highly susceptible to *Staphylococcus aureus* Infection." *The Journal of Immunology*. Vol. 165 (Nov 15, 2000): 5392- 5396.

9. Fournier, Bénédicte and Dana J. Philpott. "Recognition of *Staphylococcus aureus* by the Innate Immune System." *Clinical Microbiology Reviews.* Vol. 18, No. 3 (2005): 521-540.

10. Nograles, K.E., et al. "Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways." *British Journal of Dermatology.* Vol. 159 (2008): 1092-1102.

11. Jancàlek, R., et al. “Bilateral changes of TNF-alpha and IL-10 protein in the lumbar and cervical dorsal root ganglia following a unilateral chronic constriction injury of the sciatic nerve.” *Journal of Neuroinflammation*. Vol. 7:11 (2010).

12. Reinholz, Markus, et al. “Cathelicidin LL-37: An Antimicrobial peptide with a Role in Inflammatory Skin Disease.” *Annals of Dermatology.* Vol. 24, No. 2 (2012): 126-135.

13. Scaffidi, Paola,et al. “Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.” *Nature*. Vol. 418 (July 2002): 191-195. Web.

14. Miller, Lloyd S. and John S. Cho. “Immunity against *Staphylococcus aureus* cutaneous infections.” *Nature Immunology*. Vol. 11. (2011): 505-518.

15. DeLeo, Frank R., et al. “Community-associated methicillin-resistant *Staphylococcus aureus*.” *The Lancet.* Vol. 375 (2010): 1557-1568.

16. Cho, John S., et al. “IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice.” *The Journal of Clinical Investigation*. Vol. 120, No. 5 (2010): 1762-1773.

17. Ospelt, Caroline and Steffen Gay. “TLRs and chronic inflammation.” *The International Journal of Biochemistry and Cell Biology*. Vol. 42 (4) (2010): 495-505.

18. Lai, Yuping and Richard L. Gallo. “AMPed Up immunity: how antimicrobial peptides have multiple roles in immune defense.” *Trends Immunol*. Vol. 30, No. 3 (2009): 131-141.

19. Yu, Man, et al. “HMGB1 Signals Through Toll-like Receptor (TLR) 4 and TLR2.” *Shock*. Vol. 26, No. 2 (2006): 174-179.

20. Liu, Catherine and Henry F. Chambers. “*Staphylococcus aureus* with Heterogeneous Resistance to Vancomycin: Epidemiology, Clinical Significance, and Critical Assessment of Diagnostic Methods.” *Antimicrob Agents Chemother.* Vol. 47 (10) (2003): 3040-3045.

21. Kisich, Kevin O., et al. “The Constitutive Capacity of Human Keratinocytes to Kill *Staphylococcus aureus* is Dependent on Beta-Defensin 3.” *Journal of Investigative Dermatology.* Vol. 127 (10) (2007): 2368-2380.

22. Bonaldi, Tiziana, et al. “Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion.” *The EMBO Journal*. Vol. 22 (20) (2003): 5551-5560.

23. Lotze, Michael, T. and Kevin J. Tracey. “High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal.” *Nature Reviews Immunology*. Vol. 5 (2005): 331-342.

24. Buescher, E. Stephen. “Community-acquired methicillin-resistant *Staphylococcus aureus* in pediatrics.” *Current Opinion in Pediatrics*. Vol. 17 (1) (2005): 67-70.

25. L.E. DeForge and D.G. Remick. “Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood.” *Biochemical and Biophysical Research Communications*. Vol. 174 (1) (1991): 18-24.

26. Schlievert, Patrick M., et al. “Secreted Virulence Factor Comparison Between Methicillin-Resistant and Methicillin-Sensitive *Staphylococcus aureus*, and its Relevance to Atopic Dermatitis.” *J Allergy Clin Immunol.* Vol. 125 (1) (2010): 39-49.

27. Harris, H.E. and Ulf Andersson. “Mini review: The nuclear protein HMGB1 as a proinflammatory mediator.” *Eur. J. Immunol*. Vol. 34 (2004): 1503-1512.

28. Kollisch, G., et al. “Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes.” *Immunology*. Vol. 114 (4) (2005): 531-541.

29. Stryjewskil, Martin E. and Henry F. Chambers. “Skin and Soft-Tissue Infections Caused by Community-Acquired Methicillin-Resistant *Staphylococcus aureus*.” *Oxford Journals: Clin Infect Dis.* Vol. 46 (Supplement 5) (2008): S368-S377.

30. Johansson, Linda, et al. “HMGB1 in severe soft tissue infections caused by *Streptococcus pyogenes*.” *Frontiers in Cellular and Infection Microbiology.* Vol. 4 (4) (2014): 1-8.

31. Chen, Tao, et al. “Increased HMGB1 serum levels and altered HMGB1 expression in patients with psoriasis vulgaris.” *Arch Dermatol Res*. Vol. 305 (2013): 263-267.

32. Goldstein R.S, et al. “Cholinergic anti-inflammatory pathway activity and High Mobility Group Box-1 (HMGB1) serum levels in patients with rheumatoid arthritis.” *Mol Med.* Vol.13 (3-4) (2007): 210-215.

33. Oktayoglu, P., et al. “Elevated serum levels of high mobility group box protein 1 (HMGB1) in patients with ankylosing spondylitis and its association with disease activity and quality of life.” *Rheumatol Int.* Vol. 33 (5) (2013): 1237-31.

34. Abdulahad D.A., et al. “HMGB1 in systemic lupus Erythematosus: its role in cutaneous lesion development.” *Autoimmun Rev.* Vol. 9 (2010): 661-665.

35. Lesiak, A., et al. "The Role of T-Regulatory Cells and Toll-like Receptors 2 and 4 in Atopic Dermatitis." *Scandinavian Journal of Immunology.* Vol. 76 (4) (2012): 405-410.

36. Bardoel, B.W., et al. "Evasion of Toll-like receptor 2 activation by staphylococcal superantigen-like protein 3." *J Mol Med (Berl)*. Vol. 90 (10) (2012): 1109-1120.

37. Arcus, Vickery L, et al. "Protein Structure and Folding: The Three-dimensional Structure of a Superantigen-like protein, SET3, from a Pathogenicity Island of the *Staphylococcus aureus* Genome." *The Journal of Biological Chemistry*. Vol. 277 (35) (2002): 32274-32281.

38. Yokoyama, Ryosuke, et al. "Staphlococcal Superantigen-Like Protein 3 Binds to the Toll Like Receptor 2 Extracellular Domain and Inhibits Cytokine Production Induced by *Staphylococcus aureus*, Cell Wall Component, or lipopeptides in Murine Macorphages." *Infection and Immunity*. Vol. 80 (8) (2012): 2816-2825.

39. Schroder, JM, et al. "Who is really in control of skin immunity under physiologic circumstances – lymphocytes, dendritic cells or keratinocytes?" *Experimental Dermatology*. Vol. 15 (2006): 913-329.

40. Goodarzi, Heidi, et al. "Innate Immunity: A Cutaneous Perspective." *Clinic Rev Alerg Immunol.* Vol. 33 (2007): 15-26.

41. Penzo, M., et al. "Inhibitor of NF-kappa B kinases alpha and beta are both essential for high mobility group box 1-mediated chemotaxis [corrected]." *J. Immunol*. Vol. 184 (2010): 4497-4509.

42. Sunden-Cullbert, J., et al. "Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock." *Crit. Car Med*. Vol. 33 (2005): 564-573.

43. Novick, Richard P. "Autoinduction and signal transduction in the regulation of staphylococcal virulence." *Molecular Microbiology*. Vol. 48 (6) (2003): 1429-1449.

44. Recsei, P., et al. "Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar." *Mol Gen Genet.* Vol. 202 (1) (1986): 58-61.

45. Brown, Aisling F., et al. "*Staphylococcus aureus* Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design." *Front Immunol.* Vol. 4 (507) (2013): 1-20.

46. Hilmi, Dina, et al. "Heterogeneity of Host TLR2 Stimulation by *Staphylococcus aureus* Isolates." *PLoSONE.* Vol. 9 (5) (2014): doi: 10.1371/journal.pone.0096416

47. Shapira, S., et al. "Suppression of NF-κB Activation by Infection with *Toxoplasma gondii*." *The Journal of Infectious Diseases*. Vol. 185(Suppl 1) (2002): S66-72.

48. Brown, Aisling F., et al. "*Staphylococcus aureus* colonization: modulation of host immune response and impact on human vaccine design." *Frontiers in Immunology*. Vol. 4 (507) (2014): 1-20.

49. Davis, S. L., et al. "Epidemiology and Outcomes of Community-Associated Methicillin-Resistant *Staphylococcus aureus* Infection." *Journal of Clinical Microbiology.* Vol. 45 (6) (2007): 1705-1711.

50. McVicker, Gareth, et al. "Clonal Expansion during *Staphylococcus aureus* Infection Dynamics Reveals the Effect of Antibiotic Intervention." *PLoS Pathog.* Vol. 10 (2) (2014): e1003959. doi:10.1371/journal.ppat.1003959.

51. Chen, CS., et al. "Nasal carriage rate and molecular epidemiology of methicillin-resistant Staphylococcus aureus among medical students at a Taiwanese university." *Int J Infect Dis*. Vol. 16 (11) (2012): 799-803.

52. Noskin, Gary A., et al. "National Trends in *Staphylococcus aureus* Infection Rates: Impact on Economic Burden and Mortality over a 6-Year Period (1998-2003)." *Clin Infect Dis.* Vol. 45 (9) (2007): 1132-1140.

53. Klevans, R. Monina, et al. "Invasive Methicillin-Resistant *Staphylococcus aureus* Infections in the United States." *JAMA*. Vol. 298 (15) (2007): 1763-1771.

54. Boucher, Helen W. and G. Ralph Corey. "Epidemiology of Methicillin-Resistant *Staphylococcus aureus*." *Clin Infect D*is. Vol. 46 (Supplement 5) (2008): S344-S349.

55. Lee, B. Y., et al. "The economic burden of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA)." *Clinical Microbiology and Infection.* Vol. 19 (6) (2013): 528-536.

56. Krishna, Sheila and Lloyd S. Miller. "Innate and adaptive immune responses against *Staphylococcus aureus* skin infections." *Semin Immunopathol.* Vol. 34 (2012): 261-280.

57. Opal, Steven M. and Jonathan Cohen. "Clinical Gram-positive sepsis: Does it fundamentally differ from Gram-negative bacterial sepsis?" *Critical Care Medicine*. Vol. 27 (8) (1999): 1608-1616.

58. Alba-Loureiro, TC, et al. "Neutrophil function and metabolism in individuals with diabetes mellitus." *Braz J Med Biol Res.* Vol. 40 (2007): 1037-1044.

59. Chonchol M. "Neutrophil dysfunction and infection risk in end-stage renal disease. *Semin Dial.* Vol. 19 (2006): 291-296.

60. Molne L, et al. "Role of neutrophils leukocytes in cutaneous infection caused by *Staphylococcus aureus*." *Infect Immun.* Vol. 68 (2000): 6162-6167.

61. Bouma, G., et al. "Recent advances in the understanding of genetic defects of neutrophils number and function." *Vr J haematol*. Vol. 151 (2010): 312-326.

62. Shinefield H., et al. "Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis." *N Engl J Med*. Vol 346 (2002): 491-496.

63. Kobayashi, Scott D. and Frank R DeLeo. "Between Bedside and Bench: A MRSA-terious enemy among us – Boosting MRSA vaccines." *Nature Medicine*. Vol. 17 (2) (2011): 168-169.

64. Manfredi, R., et al. "Epidemiology and microbiology of cellulitis and bacterial soft tissue infection during HIV disease: a 10-year survey." *J Cutan Pathol*. Vol. 29 (2002): 168-172.

65. Minegishi Y., et al. "Molecular explanation for the contradiction between systemic Th17 defect and localized bacterial infection in hyper-IgE syndrome." *J Exp Med.* Vol. 206 (2009): 1291-1301.

66. Thurlow, Lance R., et al. "*Staphylococcus aureus* Biofilms Prevent Macrophage Phagocytosis and Attenuate Inflammation *In Vivo*." *The Journal of Immunology*. Vol. 186 (11) (2011): 6585-6596.

67. Shaw, Peter J. "*Handbook of Biological Confocal Microscopy."* Third edition. New York: SpringerScience+Business Media, 2006.

68. Okuma, Keiko, et al. "Dissemination of New Methicillin-Resistant *Staphylococcus aureus Clones* in the Community." *J. Clin Microbiol*. Vol. 40 (11) (2002): 4289 4294.

69. David, Michael, Z. and Robert S. Daum. "Community-Associated Methicillin Resistant *Staphylococcus aureus:* Epidemiology and Clinical Consequences of an Emerging Epidemic." *Clinical Microbiology Reviews*. Vol. 23 (3) (2010): 616-687.

70. Klein, Eili, et al. "Hospitalizations and Deaths Caused by Methicillin-Resistant *Staphylococcus aureus*, United States, 1999-2005." *Emerging Infectious Diseases.*Vol. 13 (12) (2007): 1840-1846.

71. Robinson, DA., et al. “Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired methicillin-resistant clone.” *Lancet.* Vol. 365 (9466) (2005): 1256-1258.

72. Wang, R., et al. “Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA.” *Nat Med.* Vol. 13 (12) (2007): 1510-1514.

73. Asadullah, K., et al. “IL-10 is a key cytokine in psoriasis. Proof of principle by IL-10 therapy: a new therapeutic approach.” *J Clin Invest*. Vol. 101 (4) (1998): 7830794.

74. Lai, Yuping, et al. “Activation of TLR2 by a Small Molecule Produced by *Staphylococcus epidermidis* increases Antimicrobial Defense against Bacterial Skin Infections.” *JID*. Vol. 130 (2010): 2211-2221.

75. Nilsen, Nadra, J., et al. “Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling; role of CD14 and CD36. *J of Leuko Biol.* Vol. 84 (1) (2008): 280-291.

76. Muller, P., et al. “The *Staphylococcus aureus* lipoprotein SitC colocalizes with Toll like receptor 2 (TLR2) in murine keratinocytes and elicits intracellular TLR2 accumulation.” *Infect Immun.* Vol. 78 (10) (2010): 4243-4250.

77. Voyich, Jovanka M., et al. “Insights into Mechanisms Used by *Staphylococcus aureus* to Avoid Destruction by Human Neutrophils.” *The Journal of Immunology*. Vol. 175 (2005): 3907-3919.

78. Le Maréchal, Caroline, et al. “Molecular Basis of Virulence in *Staphylococcus aureus* Mastitis.” *PLoS ONE*. Vol. 6(11) (2011): e27354.

79. Gordon, Rachel J. and Franklin D. Lowy. “Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clin Infect Dis.* Vol. 46 (2008): S350-S359.

80. *What is Deconvolution?* Purdue U, n.d. Web. 30 June 2014. <http://www.cyto.purdue.edu>

81. Zhang, Q., et al. “Role of high mobility group box 1 (HMGB1) in wound healing.” *J Surg Res.* Vol. 176(1) (2012): 343-347.

82. Schiraldi, Milena, et al. “HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4.” *JEM*. Vol. 209 (3) (2012): 551-563.

83. Wei, Tzuping, et al. “Keratinocyte expression of inflammatory mediators plays a

crucial role in substance P-induced acute and chronic pain." *Journal of Neuroinflammation*. Vol. 9 (2012): doi:10.1186/1742-2094-9-181.

VITA

Ashley Lynn Larm was born in Cadillac, MI on April, 20th 1990. In 2012 she graduated from Eastern Michigan University in Ypsilanti, MI with two Bachelor of Science degrees, one in Biology and one in General Chemistry. While at EMU she also completed a minor in language, literature and writing. As an undergraduate student Ashley participated in new student orientation, the peer tutoring program, alternative spring breaks, the honors college, and the pre-medical club.

In 2012 she began her graduate work in the Infectious Disease and Immunology program at Loyola University Chicago in pursuit of the Master of Science degree. In 2013, Ashley joined the research lab of Dr. Katherine Radek where she investigated the interactions of recurrent CA-MRSA bacteria with keratinocytes and the host innate immune system. In addition to her graduate coursework, she also volunteered as a biweekly playroom attendant in the hematology and oncology playroom at Lurie Children's Hospital in Chicago, IL. After graduation, Ashley plans to get a job in research before applying to medical school with an ultimate goal of becoming a pediatrician.